Impact of the myeloid Krüppel-like factor 4 during pneumococcal pneumonia

DISSERTATION
zur Erlangung des akademischen Grades

doctor rerum naturalium
(Dr. rer. nat.)
im Fach Biologie
eingereicht an der

Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: 04. Juli 2018

Jai Maa

For *Ma*, *Baba* and Janine

"Without imperfection, you or I would not exist"

- Stephen Hawking
(We will miss you Mr. Hawking)

ACKNOWLEDGEMENT

I have finally finished my Ph.D. project and this would not have been possible without the help from many people.

First, I would like to thank my clinic director, Prof. Dr. Norbert Suttorp for giving me the opportunity to work at the Department of Infectious Diseases and Pulmonary Medicine, Charite - Universitätmedizin Berlin. Additionally, I would like to thank Prof. Dr. Stefan Hippenstiel and Dr. Janine Zahlten for developing together with me the research questions and proceedings of my Ph.D. project. I would specially thank Prof. Dr. Stefan Hippenstiel for helping me with the extra effort whenever it was needed so. Janine, I could not have made it to Berlin and finished my Ph.D. thesis without you. For me, Berlin will always be synonymous with you. You will always remain my Godmother.

Since staying in a foreign country involves a lot of paperwork, I would remain grateful to Pamela Glowacki for help regarding all administrative issues. I would like to thank Prof. Regine Hengge for accepting me as doctoral student when I really needed my immatriculation. I would also thank her and Prof. Dr. Andreas Meisel for accepting to be my thesis reviewers. Additionally, I remain obliged to Prof. Dr. Thomas Eitinger and Dr. Natalia Tschowri for being the Chairman and member of the doctoral committee respectively. I remain indebted to Jürgen Manchot Stiftung for providing me with the financial assistance during my doctoral studies and providing me with travel grant for conferences.

I would also like to thank the people from CVK: Moritz, Daniel, Pinkus, Markus H., Ling, Iris, Sarah V., Philipp, Bert, Kai, Alex, Laura, Catherine, Sandra P., Juan, Juliane, Cüneyt, and Ute for providing me such a comfortable and happy working place. My deepest thanks also goes to the people from CCM: Aina, Denise, Bill, Ulrike, Kathrin, Kristina, Cengiz, Sarah B., Markus B., Gustavo, Sandra W., Geraldine and most importantly Birgitt for their support throughout my doctoral studies. I am also grateful to Kristina Dietert of Freie Universtät Berlin for her kind co-operation during my doctoral studies. I take this opportunity to thank former lab members, Dr. Elena, Dr. Anshu, Dr. Christin, and Dr. Jenny, Matteo, Christian for their support as long as they have been in the lab.

This work has also been possible because of the excellent technical assistance of Claudia and Doris. Their assistance has been invaluable for my doctoral studies. Valuable inputs from Dr. Leif Erik Sander and Dr. Bastian Opitz during the lab meetings helped me shape my scientific project a level higher.

My all thanks to my teachers who instigated the scientific thinking since my undergraduate studies: A.K.S Sir (Presidency College), S.G. Sir (Presidency College), B.B. Sir (CU), P.K. Sir (JU) and Amitava Gupta Sir (JU). I am very thankful to my new friends in Berlin: Meetesh, Janhvi, Madhura, Gaurav, Shilpa and my childhood friends: SDey, SDe, SChakraborty, SKRoy, Sharmistha, Saptarshi for the intercontinental chats without which I could not have done. I remain obliged to *Swami* Baneshanand*aji*, Sujit *kaku* and Biswas *kakima*, Shankar *jethu* and *jethima* for making me feel at home in Berlin.

Lastly but most importantly, I would thank my parents and my grandmother *Don*. *Ma* and *Baba*, I think thanking you both would be too less of a word of expression. You both have been that emotional support & mental strength and had kept my spirits high even in bad times. I am ever grateful for changing your sleep pattern according to European standard time. I think I do not need to go to the temple if I have you both in my life.

DECLARATION OF ACADEMIC HONESTY

I hereby declare that the work presented in this thesis done by me independently with the stated sources and equipment.

Hiermit erkläre ich, dass die in dieser Arbeit vorgestellten Arbeiten von mir unabhängig mit den angegebenen Quellen und Geräten durchgeführt werden.

Berlin, May 2018

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1 SUMMARY

Bacterial pneumonia is one of the leading causes of death worldwide. *Streptococcus pneumoniae*, the most frequently isolated pathogen from clinical pneumonia samples is responsible for 19% death of children below 5 years based on a report from World Health Organization. Despite the availability of different vaccines, *Streptococcus pneumoniae* remains the most common pathogen responsible for bacterial pneumonia throughout the world and emergence of antibiotic resistance has occurred. Thus, there is high medical need to establish new adjunctive therapeutic strategies for treatment of pneumococcal pneumonia.

In pneumonia, the tight control of the inflammatory response and the maintenance of tissue integrity are imperative for the protection of lung function and disease outcome. Severe pneumococcal pneumonia is accompanied by massive lung inflammation and bears the risk of deleterious respiratory failure as well as of systemic spread of the invading pathogens. Therefore, the innate immune response has to be on one side robust, rapid, and highly efficient to kill the pathogens but on the other side, it also has to be tightly controlled to prevent excessive tissue damage caused by pathogens and the host response itself. Neutrophils belong to the class of myeloid cells and forms an important component of this innate immune system against bacterial infections. Krüppel-like factor 4 (KLF4) has been reported to not only play a role in differentiation of cells of the immune system but also in mediating inflammatory signals in the host cells during infection in different cell types such as endothelial-, epithelial cells and cells of the innate and adaptive immunity. Taking into account that neutrophils are one of the most important regulators of innate immunity during pneumococcal pneumonia and that KLF4 has an important role in the neutrophil function during bacterial infection, the hypothesis was tested about the role of KLF4 in neutrophil function during *S. pneumoniae* induced infection in a mice model.

This study is the first time, which shows myeloid KLF4 has an impact on pneumococcal pneumonia outcome and regulates the inflammation associated with bacterial pneumonia *in vivo* in mice. The results presented in the work show that the transcription factor KLF4 is induced in human and mice neutrophils during pneumococcal pneumonia. The induction of KLF4 is time and dose dependent. Additionally, the expression of myeloid KLF4 is regulated by the autolysis of *S. pneumonia*e but is not mediated via Toll-like receptor (TLR) 2, TLR4 or TLR9.

Studies using a mouse pneumonia model showed that myeloid KLF4 exhibits a pro-inflammatory phenotype. Mice with KLF4 knockout (KO) or $KLF4^{-/-}$ in myeloid cells had

higher bacterial load in their lungs, blood and spleen in comparison to wildtype (WT) or $KLF4^{+/+}$ mice. Although there was less pro-inflammatory cytokine (such as TNF-α, IL-1β and KC) production in the broncho-alveolar lavage fluid (BALF) and plasma of $KLF4^{-/-}$ mice yet there no differences in cell recruitment in the BALF of the $KLF4^{-/-}$ and $KLF4^{+/+}$ mice. There was however less cell recruitment in the blood of $KLF4^{-/-}$ mice in comparison to $KLF4^{+/+}$ mice. Additionally, an increased vascular permeability associated with perivascular edema and pleuritis was seen during *Streptococcus pneumoniae*-induced infection in $KLF4^{-/-}$ mice, which also reached earlier the human endpoints than the $KLF4^{+/+}$ mice.

Taken together, myeloid KLF4 has a pro-inflammatory nature as a result of which $KLF4^{-/-}$ mice has a higher bacterial load with respect to $KLF4^{+/+}$ after infection with *S. pneumoniae*. The higher bacterial load is because of the less cytokine production by the myeloid cells and this higher concentration of bacteria disrupts the lung architecture and consequently causes faster progression of the disease of the $KLF4^{-/-}$ mice.

The results point out how KLF4 can modulate the immune functions orchestrated by the myeloid cells during pneumococcal pneumonia and this could help us develop host directed therapeutic options during bacterial pneumonia.

Keywords:

Krüppel-like factor 4, neutrophils, pneumonia mouse model, *Streptococcus pneumoniae*

ZUSSAMMENFASSUNG

Bakterielle Pneumonien sind weltweit eine der häufigsten Todesursachen. Basierend auf einem Bericht der Weltgesundheitsorganisation ist *Streptococcus pneumoniae* der am häufigsten in klinischen Proben nachgewiesene Erreger und zu 19% für den Tod von Kindern unter 5 Jahren verantwortlich. Obgleich mehrere Impfungen verfügbar sind, stellen Pneumokokken weltweit nach wie vor den häufigsten Erreger der Pneumonie dar und treten vermehrt Antibiotikaresistenzen auf. Daher besteht eine hoher medizinischer Bedarf für die Entwicklung neuer adjunktiver Therapiestrategien zur Behandlung der Pneumokokkenpneumonie.

In der Pneumonie sind eine präzise Kontrolle der Immunantwort und die Gewährleistung der Gewebeintegrität zwingend für den Erhalt der Lungenfunktion und einen positiven Krankheitsverlauf. Schwere Pneumokokken Pneumonien werden begleitet von massiven Entzündungsreaktionen in der Lunge und bergen das Risiko lebensbedrohlicher Atemstörungen sowie einer systemischen Ausbreitung des eingedrungenen Erregers. Deshalb muss die angeborene Immunantwort einerseits robust, schnell und hocheffizient das Pathogen ausschalten, andererseits aber so kontrolliert ablaufen, dass übermäßige Gewebeschädigungen durch den Erreger und die Immunantwort selbst vermieden werden. Neutrophile Granulozyten gehören zur Klasse der myeloiden Zellen und sind eine wichtige Komponente der angeborenen Immunität gegen bakterielle Infektionen. Krüppel-like factor 4 (KLF4) spielt dabei nicht nur eine Rolle in der Differenzierung der Zellen des Immunsystems, sondern auch während der Infektion bei der Vermittlung inflammatorischer Signale in den Wirtszellen wie Endothel- und Epithelzellen sowie Zellen der angeborenen und erworbenen Immunität. Aufgrund der herausragenden Rolle von Neutrophilen Granulozyten als Regulatoren angeborener Immunität und KLF4 als wichtigem Faktor in der Regulierung von Neutrophilen im Kontext bakterieller Infektionen wurde die Rolle von KLF4 in Neutrophilen im *S. pneumoniae Maus-Infektionsmodel untersucht.*

Diese Studie zeigt zum ersten Mal *in vivo* in mäusen, dass myeloides KLF4 Einfluss auf den Krankheitsverlauf hat und die mit einer bakteriellen Pneumonie einhergehende Entzündungsreaktion reguliert. Die hier aufgeführten Ergebnisse demonstrieren, dass der Transkriptionsfaktor KLF4 während einer Pneumokokken Pneumonie in humanen und murinen neutrophilen Granulozyten induziert wird. Diese Induktion ist Zeit- und Dosisabhängig. Außerdem wird die Expression von myeloidem KLF4 durch die Autolyse von

S. pneumoniae reguliert, aber nicht über Toll-like Rezeptor 2 (TLR2), TLR4 oder TLR9 vermittelt.

Studien in einem Maus-Pneumonie Modell zeigen, dass myeloides KLF4 einen proinflammatorischen Phänotyp bewirkt. Mäuse mit einem KLF4 *knockout* (KO) oder $KLF4^{-/-}$ in myeloiden Zellen haben im Vergleich zu Wildtyp (WT) oder $KLF4^{+/+}$ Mäusen eine höhere Bakterienlast in Lunge, Blut und Milz. Obwohl die Produktion pro-inflammatorischer Zytokine (wie TNF-α, IL-1β und KC) in der bronchoalveolären Lavageflüssigkeit (BALF) und im Plasma von $KLF4^{-/-}$ Mäusen geringer war, gab es keine Unterschiede bei der Zellrekrutierung in der BALF von $KLF4^{-/-}$ und $KLF4^{+/+}$ Mäusen. Allerdings war die Zellrekrutierung im Blut der $KLF4^{-/-}$ Mäuse geringer als bei den $KLF4^{+/+}$ Mäusen. Außerdem wurde eine erhöhte vaskuläre Permeabilität verbunden mit perivaskulären Ödemen und Pleuritis bei $KLF4^{-/-}$ Mäusen während der *S. pneumoniae*-induzierten Infektion beobachtet. Diese Mäuse erreichten auch eher die humanen Endpunkte als die vergleichbaren $KLF4^{+/+}$ Mäuse.

Zusammengefasst zeigte myeloides KLF4 einen pro-inflammatorischen Charakter, wodurch $KLF4^{-/-}$ Mäuse nach Infektion mit *S. pneumoniae* eine höhere Bakterienlast im Vergleich zu $KLF4^{+/+}$ Mäusen hatten. Die höhere Bakterienlast resultierte aus der geringeren Zytokinproduktion der Myeloidzellen, die höhere Bakterienkonzentration zerstörte die Struktur des Lungengewebes und führte so zu einem progressiveren Krankheitsverlauf in $KLF4^{-/-}$ Mäusen.

Die Erkenntnis, dass KLF4 die Immunfunktionen der Myeloidzellen während der Pneumokokken Pneumonie moduliert kann womöglich helfen, wirtsspezifische Therapien in der bakteriellen Pneumonie zu entwickeln.

Schlagwörter:

Krüppel-like factor 4, murines Pneumoniemodell, Neutrophile, *Streptococcus pneumoniae*

2 LIST OF ABBREVIATIONS

Short form	Full form
°C	Centigrade
AFR	African region
AKI	Acute kidney injury
AML	Acute myeloid leukemia
AMR	Americas region
ANOVA	Analysis of variance
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BALF	Broncho-alveolar lavage fluid
BM	Bone marrow
BMMs	Bone marrow-derived macrophages
bp	Base pairs
BSA	Bovine serum albumin
C	Control
CAP	Community acquired pneumonia
CAPNETZ	German network for community acquired pneumonia
CARD	Caspase-recruiting domain
CD	cluster of differentiation
CDC	Centers for disease control and prevention
cDCs	Classical dendritic cells

cdk	Cyclin-dependent kinase
CFU	Colony forming unit
CLR	C-type lectin receptor
c-myc	v-myc myelocytomatosis viral oncogene homologous
CO_2	Carbon dioxide
CpG	2'-deoxyribo cytidine–phosphate–guanosine
CRD	Carbohydrate recognition domain
cre	Cyclization recombination
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease
CTLD	C-type lectin-like domain
DACH1	Dachshund homolog 1
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMR	Eastern Mediterranean region
Eno	Enolase
ERK1/2	Extracellular signal-related kinases1/2
EUR	European region
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum

FELASA	Federation of European laboratory animal science associations
g	Times gravity
G-CSF	Granulocyte colony stimulating factor
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
Hyl	Hyaluronate lyase
i.p.	Intraperitoneal
ICAM-1	Intracellular adhesion molecule-1
ICE	IL-1 converting enzyme
IFNAR	Interferon-α/β receptor
IFN-β	Interferon-beta
Ig	Immunoglobulin
IL	Interleukin
IL-1R	IL-1 receptor
iNOS	inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
IκBα	Inhibitor of κB alpha
JNK	C-jun N-terminal kinase
K_2EDTA	Potassium EDTA
KC	Keratinocyte-derived chemokine / Keratinocyte chemoattractant
kDa	Kilo Dalton
$KHCO_3$	Potassium hydrogen carbonate

KLF	Krüppel-like factor
KO	Knockout
LAGeSo	*Landesamt für Gesundheit und Soziales*
LGP2	laboratory of genetics and physiology2
LoxP	Locus of X-over P1
LPDCs	Lamina propria dendritic cells
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
LTA	Lipoteichoic acid
LytA	N-acetylmuramoyl-L-alanine amidase
Lyz	Lysozyme
MA	Mouse albumin
MALP-2	Macrophage-activating lipopeptide-2
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen activated protein kinase
MDA5	Melanoma differentiated associated gene5
MDR	Multi drug resistant
$MgCl_2$	Magnesium chloride
MHC-II	Major histocompatibility complex-II
MIF	Macrophage migration inhibitory factor
MMP-9	Matrix metallopeptidase-9
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MR	Mannose receptor

mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NaCl	Sodium chloride
NCTC	National collection of type cultures
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_4Cl	Ammonium chloride
NLR	NOD-like receptor
NLS	Nuclear localization signal
NOD	Nucleotide-binding oligomerization domain
O_2	Oxygen
Oct3/4	Octamer binding transcription factor 3/4
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PavA	Pneumococcal adhesion and virulence A
PBP	Penicillin binding protein
PBS	Phosphate-buffered saline
PBST	PBS + Tween-20
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PFA	Paraformaldehyde
Pi3k	Phosphatidylinositol-4,5-bisphosphate 3-kinase

PiaA	Pneumococcal iron acquisition A
PiuA	Pneumococcal iron uptake A
Ply	Pneumolysin
PMNs	Polymorphonuclear leukocytes
PRR	Pattern recognition receptor
PsaA	Pneumococcal surface antigen A
Psp	Pneumococcal surface protein
RBC	Red blood cell
RER	Rough endoplasmic reticulum
RIG-I	retinoic acid-inducible gene-I
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
S. pneumoniae or S.p.	*Streptococcus pneumoniae*
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SEAR	South East Asian region
ShRNA	Short hairpin RNA
siRNA	Small interfering RNA
Sox-2	SRY -box2
SRY	Sex determining region Y
STING	Stimulator of interferon genes
TA	Teichoic acid
TAB	TAK1 binding protein

TACE	TNF-α converting enzyme
TAK-1	Transforming growth factor β-activated kinase-1
TEMED	Tetramethylethylenediamine
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
THY	Todd-Hewitt broth with yeast extract
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAF-6	Tumor necrosis factor receptor-associated factor-6
USA	United States of America
VCAM-1	Vascular cell adhesion molecule-1
WBCs	White blood cells
WHO	World Health Organization
WPR	West Pacific region
WT	Wildtype
ZF	Zinc fingers

3 INTRODUCTION

3.1 The human respiratory system

Respiration is a process by which oxygen is provided to the tissues in return for carbon dioxide. The human respiratory system consists of two parts. The upper respiratory conducting zone and the lower respiratory exchange zone. The upper conducting zone, responsible for the conduction of air to the lower exchange zone, consists mainly of the trachea, bronchi, bronchioles and the terminal bronchioles. The inspired air passes through the nasal passage, pharynx, larynx, and is warmed before it reaches the exchange zone. The exchange zone mainly consists of the respiratory bronchioles, alveolar ducts and alveoli. The air at the end points of the respiratory system, the alveoli, causes O_2 to enter the blood from the inspired air while CO_2 goes in the opposite direction. The blood vessel supplying blood to the alveoli and facilitating the blood exchange are the pulmonary capillaries which originates from the pulmonary artery and merges again to form the pulmonary vein. The air and blood interface at the alveoli is separated by distance 0.5µm due to the presence of the alveolar epithelium and the capillary endothelium. The alveolar epithelial cells are of two types-type I cells, which are responsible for gaseous exchange and the type II cells, which produces the surfactant responsible for reducing the surface tension. Multiple division of the respiratory tract massively increases the cross-sectional area thus facilitating gaseous exchange. In humans there are 23 divisions between the trachea and the alveolar sacs of which, the first sixteen divisions is responsible for air conduction only while the last seven divisions is responsible for the gaseous exchange[1,2].

3.2 Infections of the respiratory system

Infections of the upper respiratory system include epiglottitis, laryngitis, laryngotracheitis, nasopharyngitis, pharyngitis, rhinitis, sinusitis and tracheitis[3]. The infection and the respective inflammed area is given in table 1. The infections of the upper respiratory system are mostly viral origin with the exception of laryngotracheitis, epiglottitis and pharyngitis that are also caused by *Haemophilus influenzae* (*H. influenzae*) type b and Group-A β-hemolytic streptococci[4]. These diseases are mostly self-limiting in nature.

Table 1: Inflammation areas of upper respiratory tract infections.

Name of the infection	Main area(s) affected
epiglottitis	larynx
laryngitis	larynx

laryngotracheitis	larynx, trachea
nasopharyngitis	pharynx, nose
pharyngitis	pharynx, tonsils
rhinitis	nasal mucosa
sinusitis	paranasal sinuses
tracheitis	trachea

Lung infections are one of the major burden for the society[5]. Bronchitis, bronchiolitis and pneumonia are the main infections of the lower respiratory system. Inflammation of the bronchial tree is defined as bronchitis and bronchiolitis. With the inflammation of the bronchial tree, there is edema and excessive secretion within the bronchioles. Bronchitis is caused by bacterial infection in combination with environmental factors such as smoking while, bronchiolitis is caused by respiratory syncytial virus. Bacterial infections are the most common cause of lower respiratory system[4]. Of the different bacterial infections, bacterial pneumonia is one of the leading causes of death not only in developing countries but also in developed countries. A World Health Organization (WHO) organization report states that 19% of all deaths of children below five years of age is caused by pneumonia[6]. *Streptococcus pneumoniae* (*S. pneumoniae*) is the most frequently isolated pathogen responsible for bacterial pneumonia[7,8,9].

3.3 *Streptococcus pneumoniae*

S. pneumoniae was first isolated in 1880s by Louis Pasteur and George Miller Sternberg[10,11]. *S. pneumoniae* belongs to the family Streptococcaceae and is of genus *Streptococcus*. It is a gram positive coccus, normally forming diplococci given that the division of the bacterium occurs at a single axis[12]. The bacterium can be observed in small chains or in pairs. It is α-hemolytic, catalase negative and non-spore forming encapsulated bacterium. Characteristic colonies of *S. pneumoniae* are given in Fig.1.

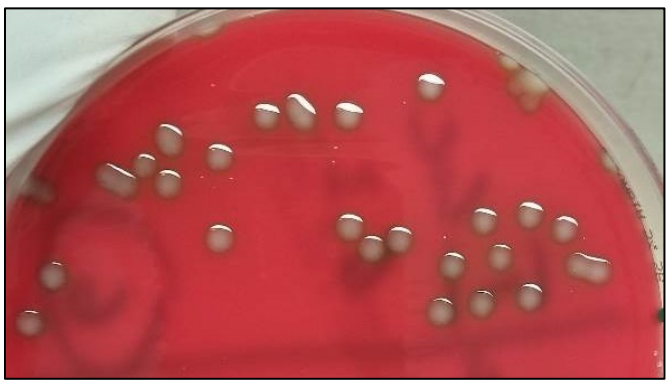


Fig. 1 Characteristic colonies of S. pneumoniae.

The α-hemolysis is an important identification for these facultative anaerobic pneumococci. Since it is catalase negative, the pneumococcal H_2O_2 changes the colour of the blood agar plates from red to green, indicating a transition of the hemoglobin to methemoglobin. Since it is facultative anaerobic, the α-hemolysis, the identification parameter of pneumococci is only possible if oxygen is present in the environment in which the bacteria is growing. However since the pneumococci is phylogenetically related to (viridans) streptococci, the α-hemolytic colonies have to be further confirmed with other tests. Other identification parameters include the optochin test and bile solubility test. Given that, other α-hemolytic streptococci are resistant to optochin (ethylhydrocupreine hydrochloride) while *S. pneumoniae* is sensitive to it, optochin tests are widely used to identify pneumococci. Similarly, with the bile solubility test, *S. pneumonia*e is bile (sodium deoxycholate) soluble whereas other other α-hemolytic streptococcal species are bile resistant[13]. Ideally, the identification of *S. pneumoniae* should be done by molecular biology methods such as by means of 16S rRNA[14]. The normal diagnostic procedure given by the centre of disease control and prevention (CDC), United States of America (USA) is given in the flowchart (Fig. 2).

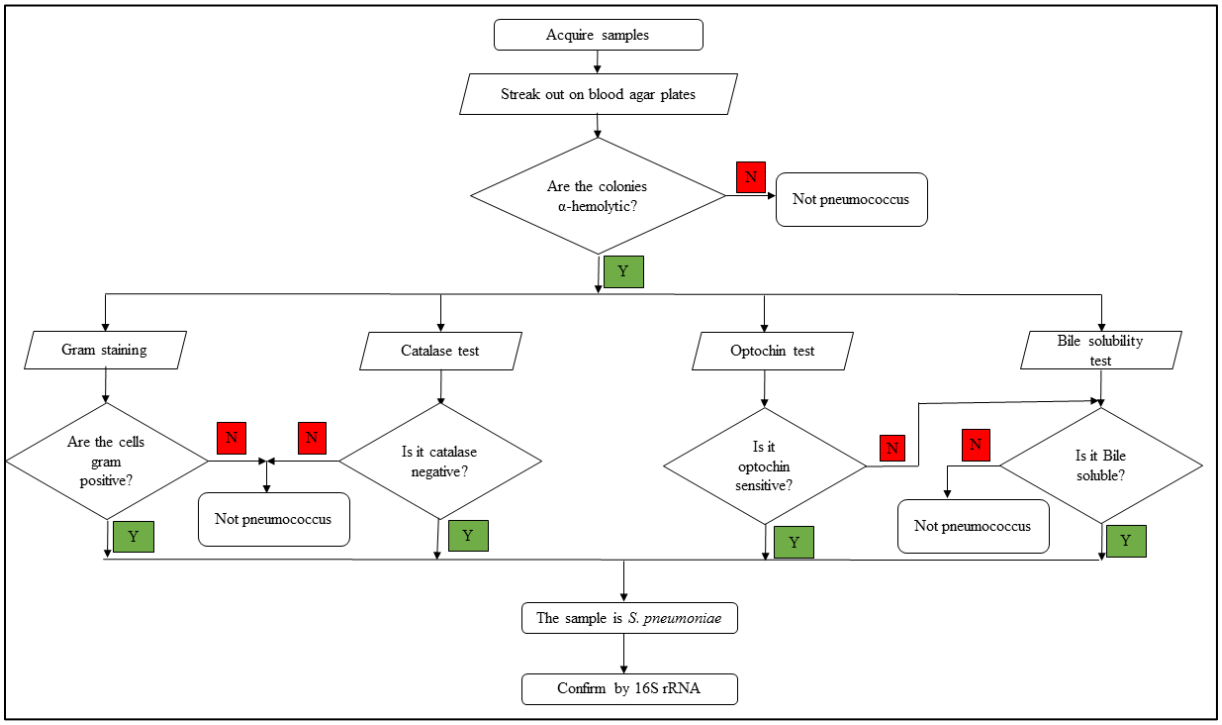


Fig. 2 Scheme for identification of S. pneumoniae.

Typical flowchart used in the detection of *S. pneumoniae*. The main test include α-hemolytic colonies on blood agar plates, gram staining, catalase test, optochin test and bile solubility test. The figure has been adapted from the CDC, USA[13]. Y= Yes, N=No.

3.3.1 Virulence factors of *S. pneumoniae*

Various virulence factors are responsible for the pathogenicity of *S. pneumoniae*. The following discussion is about the various virulence factors, which are mostly present on its surface as given and adapted in Fig. 3[15].

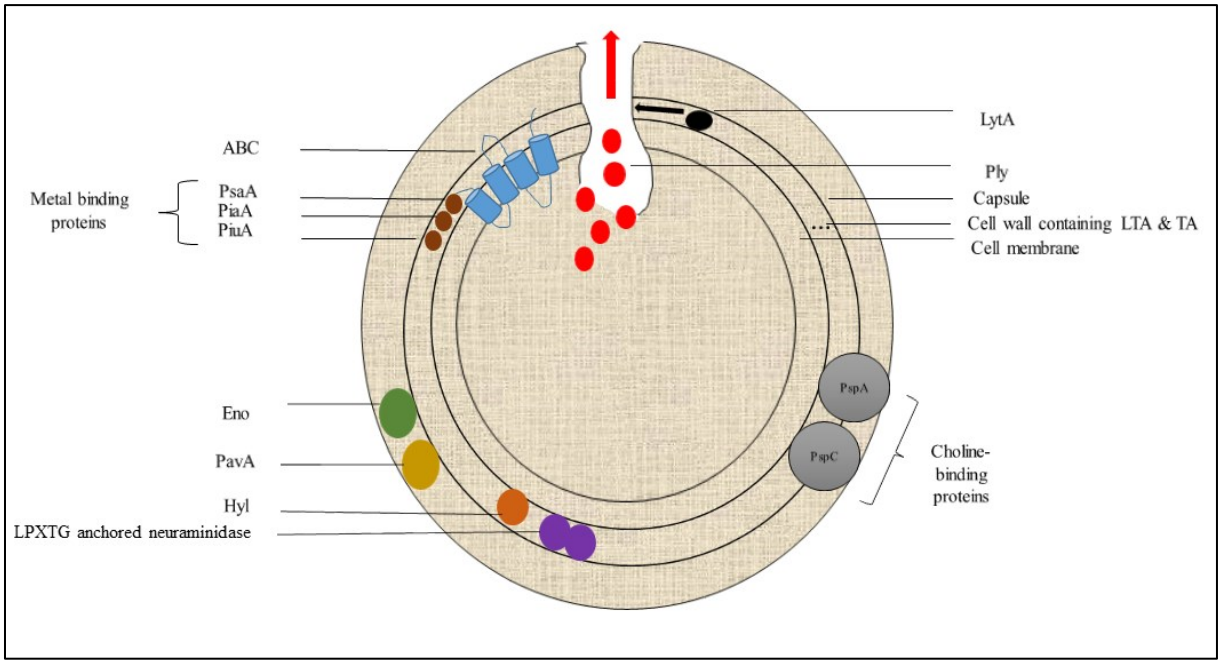


Fig. 3 The multi-faceted virulence factor of S. pneumoniae.

The various virulence factors of *S. pneumoniae* include the capsule, cell wall containing lipoteichoic acid (LTA) and teichoic acid (TA), choline-binding proteins such as pneumococcal surface protein (Psp) A and PspC, divalent metal ion binding lipoproteins such as pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA). Additional virulence factors include covalently linked proteins to the bacterial cell wall by a carboxy (C) terminal sortase (LPXTG; where X = any amino acid) motif such as neuraminidase. Other enzymatic virulence factors include hyaluronate lyase (Hyl), autolysin (LytA), pneumococcal adhesion and virulence A (PavA), pneumolysin (Ply) and enolase (Eno).

3.3.1.1 Capsule

The outermost layer of *S. pneumoniae* is its capsule. The capsule is normally 200-400 nm in thick and reports have suggested about 90 different types of structure of this capsule[16,17]. The capsule prevents the pneumococci from phagocytosis, complement deposition on the bacterial surface and reduces the chances of pneumococci from being trapped in the neutrophil extracellular traps (NETs)[15,18,19]. An interesting phenomenon is the regulation of capsule production by the *S. pneumoniae*. Previous reports have suggested that the pneumococci produce less capsular polysaccharide when it comes in close contact with the respiratory epithelial cells[20].

3.3.1.2 Cell wall associated components

Lipoteichoic acid (LTA) and teichoic acid (TA) are important cell wall components that are recognized by the host cells. TLR2 has been reported for the recognition of these cell wall components[21]. Mice lacking TLR2 has been described to show delayed bacterial clearance[22]. Literature review also suggest that LTA induce cytokine response from human mononuclear phagocytes[21]. Important characteristics of pneumococci and diseases (see below) can be induced in animal models by application of these cell wall components[23].

3.3.1.3 Surface proteins

The cell wall surface proteins of *S. pneumoniae* can be classified into three major groups: choline-binding proteins, divalent metal ion binding lipoproteins and covalently linked proteins to the bacterial cell wall by a carboxy (C) terminal sortase (LPXTG; where X = any amino acid) motif.

Of the 10-15 choline binding proteins produced by *S. pneumoniae*[24], the three main proteins include pneumococcal surface protein (Psp) A, PspC and autolysin (LytA). The three structural domain containing PspA is thought to prevent complement binding on the bacterial surface due to its high electronegative characteristics[25]. Previous publication have reported about how expression of PspA can prevent the bactericidal effect of apolactoferrin, given that PspA binds to the iron transporter lactoferrin[26,27]. PspC binds to the polymeric immunoglobulin (Ig) receptor that is responsible for the transport of secretory IgA. This interaction with the immunoglobulin receptor promotes pneumococcal adherence[28,29]. A report by Iannelli et al. have stated that PspC mutant pneumococci is much less virulent[30]. By binding with factor H, PspC also prevents the activation of alternate complement activation[15]. Pneumococcal autolysin (LytA) represent an amidase that cleaves N-acetylmuramoyl-L-alanine of the peptidoglycan present in the pneumococcal cell wall[31]. With the lysis of the cell wall, the virulence of cell wall is mediated by the release of Ply. Previous literature observed that LytA mutant *S. pneumoniae* was less virulent in a mouse pneumonia model[32].

The divalent metal ion binding lipoproteins are of various types and around 42-45 of them are encoded by the pneumococci[24]. The lipoproteins, which act as important virulence factors, include pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA). PsaA is a component of the ATP- binding cassette (ABC) which helps in manganese transport and thereby promotes pneumococcal

adherence[33,34,35]. PiaA and PiuA, as the name suggests are important components of the iron uptake system in the pneumococci. Previous reports have seen though a single mutation of either PiaA or PiuA was responsible for less virulence; however, double mutant pneumococci lacking the PiaA and PiuA, the virulence was much more reduced[36].

One of the most important virulence protein anchored by the LPXTG motif include neuraminidase[24]. *S. pneumoniae* encodes three neuraminidases genes, namely nanA, nanB and nanC[37]. This enzyme aid in the adhesion of the pneumococci to the epithelial cells by cleaving terminal sialic acid from glycoproteins, present on the host cell surface[38].

3.3.1.4 Pneumolysin

Pneumolysin (Ply) is a 52 kDa cytoplasmic toxin released by *S. pneumoniae* after autolysis of the cell wall. Ply facilitates the survival of the pneumococci in the upper and lower respiratory tract[39,40]. Ply remains as a monomer before forming oligomers which subsequently helps the pneumococci not only in inhibiting ciliary beating on epithelial cells but also in reducing respiratory bursts[41,42]. Previous reports have also suggested that the virulence factor Ply is required for the bacteria to spread from the lungs into the blood[39,40,43,44]. Ply have also been reported to have cell modulatory and compliment activation activity by which it adds to the virulence caused by the pneumococci[45,46]. Additionally, Ply has been reported to be responsible for induction of cytokine such as interleukin (IL)-1β in human lung tissue and mononuclear cells[47,48].

3.3.2 Diseases caused by *S. pneumoniae*

S. pneumoniae is responsible for various diseases. These mainly include sinusitis, otitis media, bacterial meningitis and pneumonia. The details of the main diseases is being described in brief in this section

3.3.2.1 Sinusitis

Inflammation of the sinuses and nasal passage is termed as sinusitis. Sinusitis can be either acute or chronic in nature. To determine whether the sinusitis is acute or chronic depends on the symptoms such as headache, pain over the sinuses, nasal discharge, daytime cough, which increases during the night and occasional fever. Over 60% of the cases of acute sinusitis is caused by *S. pneumoniae* and *H influenzae*[12].

3.3.2.2 Otitis media

An infection of the middle ear, otitis media can generally be classified as acute, chronic and serous. Acute otitis media in the first three months of life is caused by *S. pneumoniae* in 35-40% of the cases[12]. Common symptoms of the acute otitis media include fever, irritability, and inflammation of the tympanic membrane.

3.3.2.3 Meningitis

Acute purulent meningitis is caused by *S. pneumoniae*. Other bacterial causative agents include *H. influenzae* type b, *Neisseria meningitides*. Typical symptoms of bacterial meningitis include fever, neural dysfunction and high neutrophil count in the cerebral spinal fluid[12]. The rate of mortality associated with pneumococcal meningitis can be between 47-53%[49]. Even the survivors from pneumococcal meningitis suffer from neurological complications in comparison to other meningitis[50].

3.3.3 Pneumococcal pneumonia

Pneumococcal pneumonia is one of the leading cause of death of worldwide. *S. pneumoniae* is the most common causative agent responsible for Community acquired pneumonia (CAP). Around 60-70% of bacterial CAP reported to be caused by pneumococci[51]. Data from the German Network for Community Acquired Pneumonia (CAPNETZ) has suggested as *S. pneumoniae* as the main causative agent of pneumonia with 80% of all the cases being admitted to the hospital[7]. While in some European and worldwide meta-analysis reports have stated that incidence of *S. pneumoniae* induced CAP varies between 19.3% and 27.3% respectively[52,53]. In spite of the variations in the reported data, *S. pneumoniae* remains the most common pathogen responsible for CAP. The common clinical symptoms include shortness of breath, tachypnea, high fever, pleuritic chest pain and huge amount of purulent sputum[54]. Pneumonia is type of severe inflammatory disease marked by increase cytokine levels in blood and also in lung tissue (see below)[55,56]. There are around 2 million deaths of children below 5 years of age which may be due to the high inflammation associated with the disease. A WHO report by Rudan et al., stated that pneumonia is one of the largest cause of death in children below 5 years of age in all the WHO zones, namely; Americas region (AMR), African region (AFR), European region (EUR), Eastern Mediterranean region (EMR), South East Asian region (SEAR) and West Pacific region (WPR) as given in Fig.4[6].

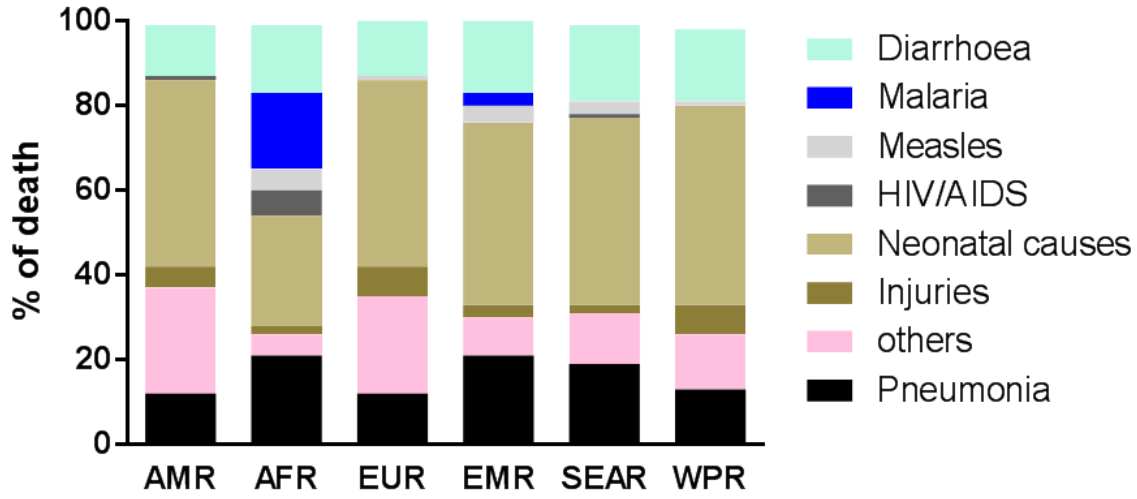


Fig. 4 Distribution of death of children below 5 years of age due to pneumonia and other causes in all the WHO zones.

Pneumonia being one of the highest cause of death of children below 5 years of age. Americas region (AMR): 12%, African region (AFR): 21%, European region (EUR): 12%, Eastern Mediterranean region (EMR): 21%, South East Asian region (SEAR): 19% and West Pacific region (WPR): 13%. The figure has been adapted from Rudan at al[6].

3.4 Emergence of multi drug resistant (MDR) *S. pneumoniae* and present vaccination strategies to prevent pneumococcal pneumonia

The emergence of MDR *S. pneumoniae* is increasing throughout the world especially with respect to macrolides and β-lactams. There have also been reports with fluoroquinolone resistance[57]. The emergence of resistance of *S. pneumoniae* to these group of antibiotics have been reported due to their wide spread improper use[58]. The emergence of resistance has led to increased mortality, morbidity and economical burden[59]. The mechanism of resistance reported to be of various causes. The macrolide resistance is because of the ribosomal methylase that dimethylates the target site of the macrolides-23S rRNA[60]. Additionally, macrolide resistance is because of the macrolide efflux pump[61]. Regarding the β-lactam resistance, it is reported about the alteration of penicillin binding proteins (PBPs) that reduced the affinity to β-lactams[62]. Given that the target of fluoroquinolones is mainly bacterial type II topoisomerase, mutation in the topoisomerase is responsible for resistance against fluoroquinolones[63].

Two types of vaccines are currently in use: the polysaccharide vaccine containing 23 different serotypes previously used in clinical scenario. However, the main disadvantage of this

polysaccharide vaccine is it cannot protect the host against pneumococcal infection. It can however reduce the chances of bacteremia[64]. The other vaccine that is presently in use is the pneumococcal conjugate vaccines (PCV). This vaccine better known as PCV-7 as it contains seven different types of pneumococcal polysaccharide (4, 6B, 9V, 14, 18C, 19F and 23F) that are most frequently isolated during pediatric infections. Though the PCV-7 seems to be promising, but there have been reports of invasive pneumococcal disease due to the serotypes not covered by the PCV-7[65]. Advancement of vaccination strategy led to the introduction of PCV-13 vaccine, which contains thirteen different serotypes of the pneumococcal capsule, however the vaccine effectiveness of PCV-13 against serotype 3 (the same serotype used in my *in vivo* project, see table 12) remains questionable[66]. Overall, the emergence of antibiotic resistance and availability of principally useful, but still insufficient vaccines, results in high medical need for the generation of adjunctive therapies by better understanding of the pathogenesis of *S. pneumoniae*-induced inflammation

3.5 The innate immune system

The body needs immune responses to protect itself from infections and invading pathogens. The immune system can be broadly classified into the cells of the innate immune system (relevant to this thesis) and the cells of the adaptive immune system. The difference between the innate immunity and adaptive immunity is that innate immune system is instantly ready for the host defense as soon as the host is attacked by the pathogen, i.e. they do not need previous exposure to the pathogens. On the other hand, the adaptive immunity in most of the times is initiated after few days of exposure of the cells to the antigens within the host. The innate immune system consists of physical barriers, which are the body's first line of defense, e.g. skin or also of chemical barriers like acid pH. Cells of the innate immune system include granulocytes (eosinophils, basophils and neutrophils), monocytes, macrophages, natural killer cells and the mast cells. The macrophages along with the neutrophils are the cells mainly responsible for phagocytosis (see below, 3.5.1). Additionally, mast cells (like the neutrophils) contain various type of granules which when released due to stimulation, adds to the local inflammation. Certain cells such as DCs are responsible for linking the innate immunity with the adaptive immunity. The adaptive immunity consists of the T cells and B cells. T cells can be of two types, CD4 T cells, which regulate the adaptive immune responses, and CD8 T cells, after developing into cytotoxic cells can kill cells infected with the microbes. B cells are responsible for secreting antibodies[67,68].

3.5.1 Neutrophils

Neutrophils, the major immune cells during inflammation, are produced from myeloid precursors in the bone marrow by a process called granulopoesis. Once produced, which is under the control of granulocyte colony stimulating factor (G-CSF)[69], the neutrophils are subjected to multi-step maturation process. The steps of maturation include from myeloblast to promyelocyte followed by myelocyte, which further matures to form metamyelocyte to band cell and finally the polymorphonuclear leukocytes (PMNs)[70]. During maturation, the neutrophil attain three type of granules (an important characteristic of mature neutrophils) which contains pro-inflammatory proteins. The total number of proteins in the granules is greater than 700[71].The content of these granules vary from mice to human, for example the primary azurophilic granules in human contain defensins which are absent in mice[72]. The number of circulating neutrophils also vary between mice and human: in mice it is 10-25% of circulating leukocytes while it is 50-70% in human circulating leukocytes[73,74].

Various modes of defense strategies have been developed by the neutrophils to sense bacteria and in turn evoke an antibacterial response. The recognition of bacterial components by the innate immune system is discussed in a different section (see below, 3.5.2). Neutrophil recruitment at the site of infection, known as neutrophil extravasation involves the following steps: tethering, rolling, adhesion, crawling and transmigration. Inflammatory mediators such as cytokines and chemokines gradient (released by the tissue resident leukocytes after recognizing the microbe) mediate such changes by an induction of adhesion molecules such as E-selectin which in turn starts the process of neutrophil tethering[75,76,77]. Once the neutrophils reach the site of infection, they have various ways for being the antimicrobial effector. These primarily include phagocytosis and NETs or release of granule components. Phagocytosis is a process where particles having a size greater than 0.5µm (such as bacteria) are engulfed by the cells. Neutrophils are key phagocytes. The process of phagocytosis is a complex process, which begins with the pathogen binding to the cell surface of the phagocyte. With the binding of the pathogen on the cell surface, there is an induction of rearrangement of actin cytoskeleton within the phagocyte. As a result, the phagocyte progressively engulfs the pathogen and forms a vacuole known as phagosome. This phagosome fuses with the lysosome, which digest the microbe. The process of phagocytosis is faster if the cells are pre-activated or in the presence of IgG antibodies or by the complement system[78,79,80]. NETs formation includes the release of chromatin DNA and granules into the extracellular space[81]. The main function of the NETs is to trap the pathogen. A previous study has shown that blocking of NET formation aggravated dissemination of the bacteria (*Escherichia* (*E.*) *coli*) to

the distant organs[82]. Other than the chromatin DNA and the histones, there are various proteins, which form important part of the NET. These proteins also have antibacterial effects. Examples of these proteins which not only forms part of the NET but also has different antimicrobial properties include: lactoferrin which inhibit iron uptake by the pathogens, cathelicidins or its cleaved product LL-37 which disrupts the bacterial membrane and are also pro-inflammatory mediators[75,78,83]. Additionally, neutrophils interact with other cells of the immune system to orchestrate to the host defense against the microbes and can modulate the adaptive immune system. Various reports have suggested that the neutrophils can suppress T cell proliferation and activate splenic B cells[84,85,86]. There also exists a positive feedback loop mechanism between the neutrophils and the T helper 17 (Th_{17}) cells, whereby Th_{17} increases neutrophil recruitment by release of Interleukin (IL) – 17. The recruited neutrophils in turn recruit Th_{17} cells via release of different chemokines[87].

Given that lung contains a population of mature neutrophils[88], these neutrophils play an important role in the host defense against *S. pneumoniae* infection. Report have suggested as neutrophils being the main phagocytes in an acutely inflamed lung during pneumococcal pneumonia[89]. The phagocytic ability of neutrophil during bacterial pneumonia is dependent on the production of proteases which kills the ingested bacteria[90]. Additionally neutrophils also produce reactive oxygen species (ROS) for elimination of *S. pneumoniae*. Selected depletion of neutrophils have also shown that the host is not able to clear the bacterial burden of *S. pneumoniae*[91,92]. However, one such report suggest that *S. pneumoniae,* though can be trapped in NETs, but production of endonuclease by *S. pneumoniae* helps in the degradation of the NETs and in turn causes the evasion of pneumococci from the lungs into the blood[93]. Additionally, *S. pneumoniae* not only can evade NETs but also it has developed various ways to detoxify the ROS produced by the neutrophils for killing it. *S. pneumoniae* can produce NADPH oxidase, thiol peroxidase, superoxide dismutase and alkyl hydroperoxidase for clearing ROS[94].

3.5.2 Recognition of the invading bacteria

Cells of the innate immune system, which include the neutrophils and macrophages, recognize the pathogen related structure known as pathogen associated molecular patterns (PAMPs).

3.5.2.1 Pattern recognition receptors (PRRs)

The recognition of these PAMPs by the cells of the innate immune system is done by the pattern recognition receptors (PRRs). PRRs can be of different types: Toll-like receptors (TLRs), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), c-type lectin receptors (CLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and others. Given that TLRs have one of the most important role in the recognition in *S. pneumoniae*, it will discussed in a different section (3.5.2.2). NOD1 and NOD2 are the two main members of the NLRs. NOD1 recognizes the specific structures of the peptidoglycan of gram negative bacteria, NOD2 recognizes muramyl dipeptide , an important component of the peptidoglycan found in all gram positive and gram negative bacteria[95,96]. While NOD2 is responsible for recognition of bacteria such as *S. pneumoniae* and *Mycobacterium* (*M.*) *tuberculosis*, NOD1 recognizes bacteria such *E. coli*, *Pseudomonas aeruginosa*, *H. influenzae*[97]. Normally with the activation of NLRs, it leads to production of cytokines (see below) which contributes to the host defense[98,99].

CLRs recognizes the carbohydrates through the carbohydrate recognition domain (CRDs) or through the C-type lectin-like domain (CTLD) for recognition of non-carbohydrate ligands[100]. Of the 17 groups of CLRs, the mannose receptor (MR) has been identified in the recognition of bacteria such as *S. pneumoniae*, *Klebsiella pneumoniae*, *M. tuberculosis*[101]. Activation of the CLRs lead to phagocytosis, activation of respiratory bursts and production of mediators of inflammation[102].

The RLRs consists of three main members, which include the RIG-I, melanoma differentiated associated gene5 (MDA5) and laboratory of genetics and physiology2 (LGP2). The RLRs are mostly responsible for recognition of viral RNA[103,104].

3.5.2.2 Toll-like receptors (TLRs)

Toll-like receptors are mainly transmembrane receptors that consists of an extracellular domain and cytosolic domain. The extracellular domain contains leucine-rich repeats (LRRs) that are used in the recognition of PAMPs[96]. Till date 13 TLRs have been identified in mice while there are 10 members of the human TLR family[105]. The TLRs are either located on the plasma membrane such as TLR1, 2, 4, 5, 6, 11 or intracellular vesicles such as TLR3, 7, 8, 9. Components of the cell wall such as TA and LTA are recognized by TLR2[21]. TLR2 heterodimerizes with TLR1 or TLR6 and can recognize a wide range of microbe-associated molecular patterns (MAMPs) and PAMPs[106]. TLR4 is important for the recognition of LPS, which is a major component present in the gram negative bacteria[105]. Additionally, TLR4 may also be responsible for the recognition of Ply after the autolysis of pneumococci[107]. Lamina

propria DCs (LPDCs) of the small intestine express TLR5 which recognizes the flagellin, an important component of the bacterial flagella[108]. This recognition of LPDCs promotes the differentiation of naive B cells into IgA producing plasma cells[109].

TLRs localized internally are responsible for detection of nucleic acid. TLR3 and 7 is mainly responsible for detection of viral components such as RNA[110,111]. TLR8 recognizes RNA from human immunodeficiency virus (HIV)[112]. TLR9 recognizes the bacterial DNA containing unmethylated 2'-deoxyribo cytidine–phosphate–guanosine (CpG)[113]. *S. pneumoniae* is recognized by the innate immune system by various PRRs such as TLR2, TLR4 and TLR9. Additionally, NOD2 is also responsible for the recognition of *S. pneumoniae* components within the cytosol[105].

3.5.3 Downstream pathways of the PRRs

Various downstream signaling pathway are involved after the activation of the PRRs (namely the NLRs, RLRs and TLRs) which contributes to the host defense[96,109,114]. The regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen activated protein kinases (MAPK) by the PRRs will be discussed here as it is important for the thesis.

Activation of the MAPK pathway involves three adaptor molecules: p38, extracellular signal-related kinases1/2 (ERK 1/2) and c-jun N-terminal kinases (JNKs). TLRs evoke biological responses through the adaptor molecules. On such adapter molecule is myeloid differentiation primary response 88 (MyD88). MyD88 in turn recruits and forms a complex with other adapter molecules such as IL-1 receptor-associated kinases (IRAKs). The activated IRAKs interact with tumor necrosis factor receptor-associated factor (TRAF) -6. This TRAF-6 then interacts with transforming Growth Factor β-activated Kinase 1 (TAK1), TAK1 binding protein (TAB) 1, TAB2/3 complex that is the upstream regulator of MAPK. Once activated the MAPK pathway is responsible for regulating the transcription of various inflammatory genes[115,116,117].

The TLRs via the MyD88-dependent or the independent pathway may activate NF-κB[108,118]. The activation of NF-κB pathway is a multi-step process. Like the MAPK pathway, the adaptor molecule MyD88 after interaction with TRAF-6 and sequentially with TAK1, TAB1, TAB2/3 complex causes the phosphorylation of inhibitor of κB alpha (IκBα). When the NF-κB pathway is not activated, the IκBα remains bound to other members of the NF-κB family: RelA (p65) and p50. After its phosphorylation, IκBα is subjected to proteasomal degradation. The p65-p50 heterodimer then translocates to the nucleus where it control the production of inflammatory genes such as cytokines and chemokines[119].

3.5.4 Cytokines and chemokines

Small secretory proteins known as cytokines and chemokines regulate immune responses in the body. The cytokines can be divided into four different classes. The classification is based on the type of immune response induced by the cytokine which are as follows: cytotoxic, humoral, cell-mediated or allergic[120]. In this section, the different types of cytokines and chemokines, which are important for this thesis will be discussed.

Tumor necrosis factor or TNF are of two types - TNF-α produced by neutrophils, macrophages, natural killer cells and TNF-β which is a lymphocyte derived necrosis factor. TNF-α is a heterodimer produced as a 26 kDa protein[121]. This protein is transported thereafter to the cell surface via the RER and the Golgi complex[122]. At the plasma membrane, TNF-α forms non-covalent trimers[123,124] and is cleaved by TNF-α converting enzyme (TACE) to form a 17 kDa soluble form of TNF-α[125]. Trimers of this soluble form of TNF-α binds to specific TNF receptors (TNFRs) –I/II and internalized to produce the pro-inflammatory signaling such as IL-12[126]. Literature review have suggested that TLR2 and 4 are responsible for the induction of TNF-α production and this TNF-α induces e.g. the expression of adhesion molecules such as E-selectin, intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 to increase the influx of granulocytes into the site of inflammation[120]. Additionally, TNF-α has also been found to play role in the adaptive immunity as literature review suggest that it is important for the production of B cell-attracting chemokine-1 and secondary lymphoid tissue chemokine[127]. TNF-α has been reported to protect the host during pneumococcal infection[128,129].

IL-1 family consists of eleven members of which IL-1β is the most potent pro-inflammatory cytokine. Stimulation of NLRs and TLRs induces the production of IL-1β from various cell types such as neutrophils, macrophages, monocytes and hepatocytes[130,131,132]. IL-1β is produced as precursor, which subsequently is cleaved by the IL-1 converting enzyme (ICE)[133]. The active IL-1β binds to the IL-1 receptors (IL-1R) 1 or 2 and this in turn activates the MyD88 signaling pathway, which further controls the NF-κB and MAPK pathways. Like the TNF-α, IL-1β upregulates E-selectin, ICAM-1, VCAM-1 for neutrophil recruitment and activation of T-lymphocytes[119,120].

IL-10 is a potent anti-inflammatory cytokine produced by lung epithelial cells, T cells, B cells and by macrophages. The production of IL-10 has been reported to be dependent on the NF-κB pathway regulation by TLRs and NLRs. Given its anti-inflammatory nature, it reduces the

inflammation by inhibiting production of TNF-α, IL-1β, IL-6, and IL-8 in mononuclear phagocytes[120,134,135,136].

Chemokines are small proteins having three to four conserved cysteine residues. Their average molecular weight varies between 8-12 kDa and until date, 44 chemokines have been identified in the human genome[137]. These 44 different chemokines can subdivided into four different families: C-X-C, C-C, C, CX3C based on the position of the cysteine residues at the N-terminus. The C-X-C has two cysteine residues separated by an amino acid (X = amino acid), the C-C subfamily has two cysteine residues side by side, the C subfamily has only one cysteine residue and the CX3C has three amino acids between the cysteine residues (X3 = 3 variable amino acids)[138,139]. The chemokines bind to G-protein coupled receptors to activate leukocytes for an immune response[140]. Additional functions of the chemokines include cytokine expression of CD4+ T cells and differentiation of this T cells into Th1 cells[141]. Previous reports have suggested that important neutrophil genes such as keratinocyte derived chemokine (KC, the murine homolog of IL-8 in humans[142,143]) contains NF-κB binding sites in their 5' untranslated region[144]. Neutrophils are released from the bone marrow into the peripheral circulation in response to infection. This release of neutrophils from the bone marrow into the circulation is under the control of KC where KC binds to CXC chemokine receptor 2 for the egress of neutrophils into the circulation[145]. The released neutrophils are important for the eradication of pneumococci by means of phagocytosis (see 3.5.1)

Reports have also suggested that severe progression in CAP is associated with high levels of cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12[146,147,148].

3.6 Krüppel-like factor (KLFs)

Krüppel–like factors (KLFs) are zinc finger containing transcription factors in which the word "Krüppel" gets its name due to its homology to *Drosophila melanogaster* Krüppel protein. Loss of Krüppel in the fruit fly leads to a cripple (German: Krüppel) appearance of the larvae[149]. KLFs are involved in the regulation of inflammatory response, tissue homeostasis and regeneration in different cell types as well as various human diseases[150,151,152].

Till date seventeen KLFs have been identified with a recent prediction for the 18th KLF. These 17 KLFs can be subdivided into 3 groups based on their physiological roles- Group 1 includes KLF3, KLF8 and KLF12 while KLFs 1,2,4,5,6,7 fall under group 2 and Group 3 comprises KLFs 9,10,11,13,14,16 depending on their activation or repression activity while the activity of KLF15 and KLF17 remains unclear[152]. Given the role of KLF4 in

pathophysiology of lung diseases and also its role to in myeloid cells such as macrophages to promote inflammation[152], it will discussed in details in this thesis.

3.6.1 Krüppel-like factor 4

Krüppel like factor 4 (KLF4), was first found in the gut epithelial cells. KLF4, like most of the other members of the KLF family has four domains which includes a) N terminus acidic activation domain, followed by b) Repressor domain, c) Nuclear localization signal (NLS) and finally d) DNA binding domain at the C-terminus which contains Cys_2His_2 zinc finger motifs[153,154] as given Fig. 5. This sequence of KLF4 consists of 483 amino acids in mouse and has 91% homology to the human KLF4[155].

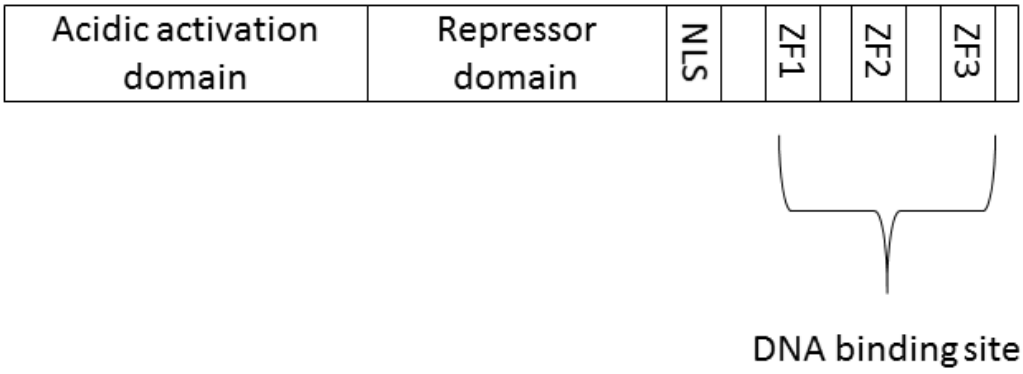


Fig. 5 Structure of the KLF4 transcription factor.

The KLF4 transcription factor consists of N-terminus acidic activation domain, which is adjacent to the repressor domain. The C-terminus domain consists of the zinc fingers (ZF) motifs and the NLS is located between the repressor domain and zinc finger motifs, which act as the DNA binding site. The figure has been adapted from Ghaleb et al.[154].

3.6.2 Functional role of KLF4

KLF4 has been documented to have a role in the establishment of the skin barrier. Given that it helps in the development of skin barrier, KLF4 KO mice die shortly after birth due to spontaneous loss of body fluids[156]. KLF4 is an important regulator of the cell cycle. Using adenoviral overexpression of KLF4 in a cell line, it could be shown that KLF4 is required for the transition from G_1 to S phase during cell cycle[157]. KLF4 achieved a further level of recognition for a scientific publication that KLF4 along with octamer binding transcription factor 3/4 (Oct3/4), sex determining region Y (SRY) -box2 (Sox2) and v-myc myelocytomatosis viral oncogene homologous (c-myc) can transform embryonic or adult fibroblast cells into pluripotent stem cells[158,159]. John B. Gurdon and Shinya Yamanaka were

awarded the Nobel Prize for Medicine in 2012 for this finding. Previous report by Hu et al. suggested that KLF4 is also down regulated in lung cancer patients, both at RNA level and protein level[160]. Given that it was already established that KLF4 had a role in cell cycle regulation, Hu et al. hypothesized that KLF4 mediated its action by regulating p21 and cyclin D1. With the activation of p21 and inhibition of cyclin D1, KLF4 induced G_1 phase arrest in lung cancer cells. A recent study by Khalife et al. has also claimed that KLF4 is a potential biomarker for patients with advanced lung cancer, which also partly echoed the findings by Hu et al.[161]. Feinberg et al. had observed that KLF4 influences monocyte differentiation and induce macrophage fate[162].

Though various reports suggest about the importance of KLF4 for various cellular functions, however there remains discrepancy about the functional role of KLF4 in myeloid cells. Recent work by Liao et al. suggests a role of KLF4 in polarization of macrophages. Interestingly, they and others observed that KLF4 expression significantly increased in M2 and decreased in M1 macrophages[163,164]. However, another study proclaim that KLF4 directs macrophages to a more pro-inflammatory M1 phenotype[165] further emphasizing that this is an area of intense moving research.

Various reports have suggested that KLF4 can regulate the NF-κB pathway in different cells especially during bacterial infection. In a recent publication, it was reported KLF4-deficient neutrophils had impaired bacterial killing and reduced cytokine production *in vivo* during *E. coli* infection. In this report, Shen et al. had also stated that the impact of KLF4 KO during the *E. coli* infection was due to the impaired NF-κB pathway[166]. KLF4 indeed regulates NF-κB in myeloid cells have long been reported when it was shown that KLF4 by interacting with the RelA complex to regulate inducible nitric oxide synthase (iNOS), an activation marker of macrophages[165]. Zahlten et al. have also reported how KLF4 can regulate the inflammation through the NF-κB pathway, during bacterial infection[136,167] (see below, 3.7).

3.7 KLF4 during pneumococcal pneumonia

Previous studies could show that KLFs could regulate the inflammation associated with pneumococcal pneumonia. Literature review shows that the induction of KLF2 in broncho-epithelial cells was initiated by the recognition of bacterial cell wall components of *S. pneumoniae* by TLR2 as well as NOD2 and the activation of Phosphatidylinositol-4,5-bisphosphate 3-kinase (Pi3K). A knockdown of KLF2 via KLF2 specific siRNA enhanced the *S. pneumoniae*- induced IL-8 secretion, which leads to the assumption that KLF2 counter-

regulates the pro-inflammatory immune response[168]. In two different publications, Zahlten et al. have pointed out that the induction of KLF4, which is highly related to KLF2, was completely independent of TLR2 or NOD2 and Pi3K activation. The expression of KLF4 in bronchial epithelial cells in contrary depends on the pneumococcal autolysin LytA related release of bacterial DNA, the activation of the endosomal TLR9 and Src kinases. Once expressed KLF4 binds to the *il10* and the *il8* promotors and leads to the production of the anti-inflammatory cytokine IL-10 and the inhibition of the pro-inflammatory chemokine IL-8 and the cytokine IL-1β whereas IL-6 remains unaffected. The inhibition of IL-8 depends on (i) direct protein-protein interaction with the NF-κB subunit p65 and (ii) the recruitment of the histone acetylase P300/CBP-associated factor (PCAF) from p65 to KLF4 thereby inhibiting the activity of NF-κB[136,167].

Herta et al. investigated the *S. pneumoniae*-related expression and the function of KLF4 in macrophages. Interestingly, strong differences between the induction and function of *S. pneumoniae*-related KLF4 expression in macrophages compared to human lung epithelial cells were found. KLF4 induction by pneumococci in macrophages seems to be tightly controlled and need the following non-redundant signals: (i) viable bacteria, (ii) LytA-related release of bacterial DNA, (iii) direct contact of viable bacteria to the host cells. At least in part the effect was mediated by TLR9 and MyD88. Noteworthy, non-CpG DNA (e.g. human, mice) which does not activate TLR9 could act as a co-signal in this KLF4 induction. Since experiments with cells derived from stimulator of interferon genes (STING)- and apoptosis-associated speck-like protein containing a CARD (ASC)-, TLR2-, TLR3-, TLR4-, TLR7-, NOD2-, Interferon-α/β receptor (IFNAR)-KO mice showed no effect on *S. pneumoniae* related KLF4 induction, the working group is convinced that another hitherto unknown DNA receptor participated in this signalling pathway. The results suggest that KLF4 induction in macrophages maybe caused by a combination of pathogen-associated molecular pattern-damage-associated molecular pattern (PAMP-DAMP) signalling and may lead to pro-inflammatory M1 phenotype[169]. Fig. 6 gives a summary of the main findings in macrophages.

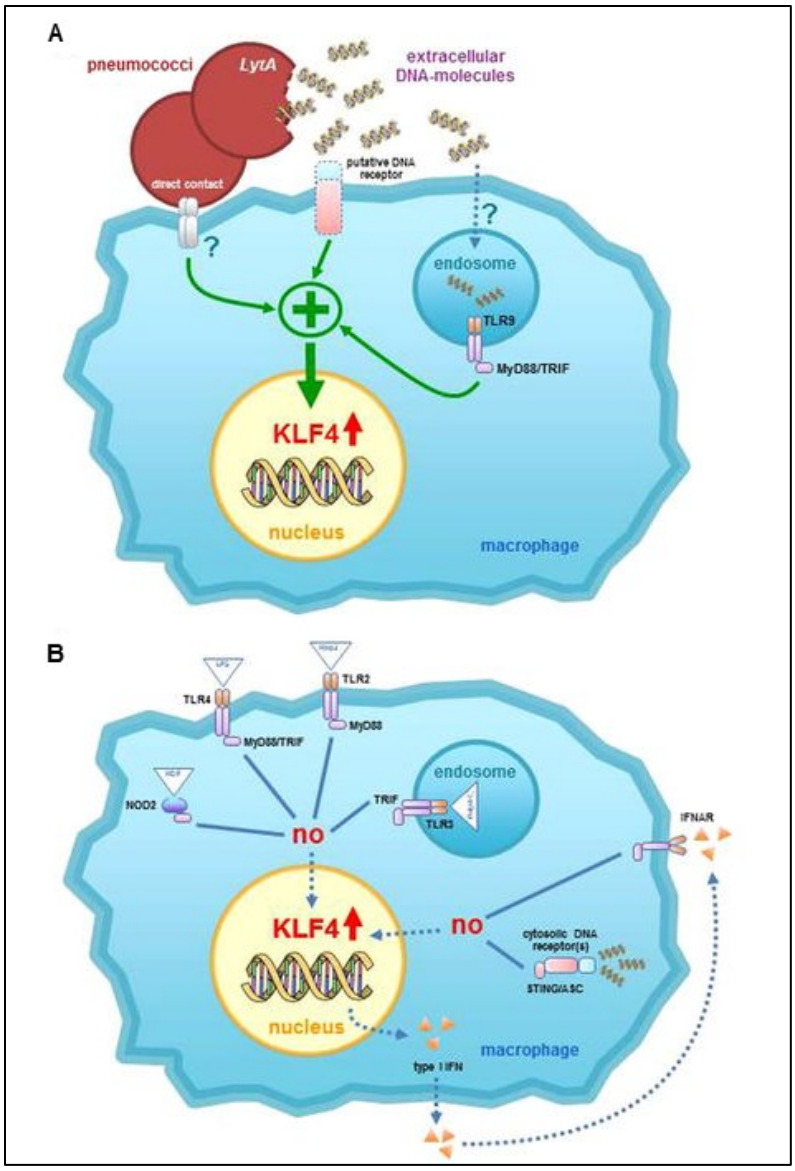


Fig. 6 Pneumococci-dependent induction of KLF4 in macrophages.

A. The induction of KLF4 expression needs direct contact of viable pneumococci to the bone marrow-derived macrophages (BMMs). During replication, the *S. pneumoniae* autolysin LytA cause the release of bacterial DNA. Free DNA is necessary for a) partly KLF4 induction via TLR9/MyD88 and b) a probably yet unknown extracellular DNA sensor.

B. Participation of TLR2, TLR4, NOD2, IFNAR and adaptor proteins such as STING, ASC are not involved in the induction of KLF4 in macrophages. Additionally, phagocytosis is also not required though TLR9 is involved in the induction of KLF4.The figure is taken from Herta et al.[169] under Creative Commons Attribution 4.0 international license.

3.8 The LyzMcre system for conditional knockout in mice

The bacteriophage P1 derived *loxP/LyzMcre* recombination system was used to create a myeloid KLF4$^{-/-}$ lineage on a C57BL/6 mice background. The *cre* (cyclization recombinase) is a site-specific DNA recombinase and can recombine DNA only at specific sites known as *loxP* (locus of X-over P1) sequences. The *cre* catalyzes the sequences spanning between two

loxP sites. As a result, since the *cre* 'sits' between the two *loxP* sites in the specific cell types only (the cells containing the *LoxP* in their genome) it knockouts the gene of interest in specific cells.

Transgenic mice containing *loxP* (which have been introduced in the embryonic stem cells by homologous recombination) are mated with transgenic mice containing *cre* gene that can be expressed in one specific cell type (LyzMcre for the myeloid cells only). The offspring from this mating will result in mice that will have both *cre* and *loxP*. As a result, cell specific LyzMcre will cause a specific gene knockout (KLF4 in this case) in myeloid cells only. This report by Clausen et al. had suggested that there can be a conditional knockout of 83-98% in mature macrophages and 100% in mature neutrophils with the LyzMcre system[170]. It is briefly illustrated in Fig. 7.

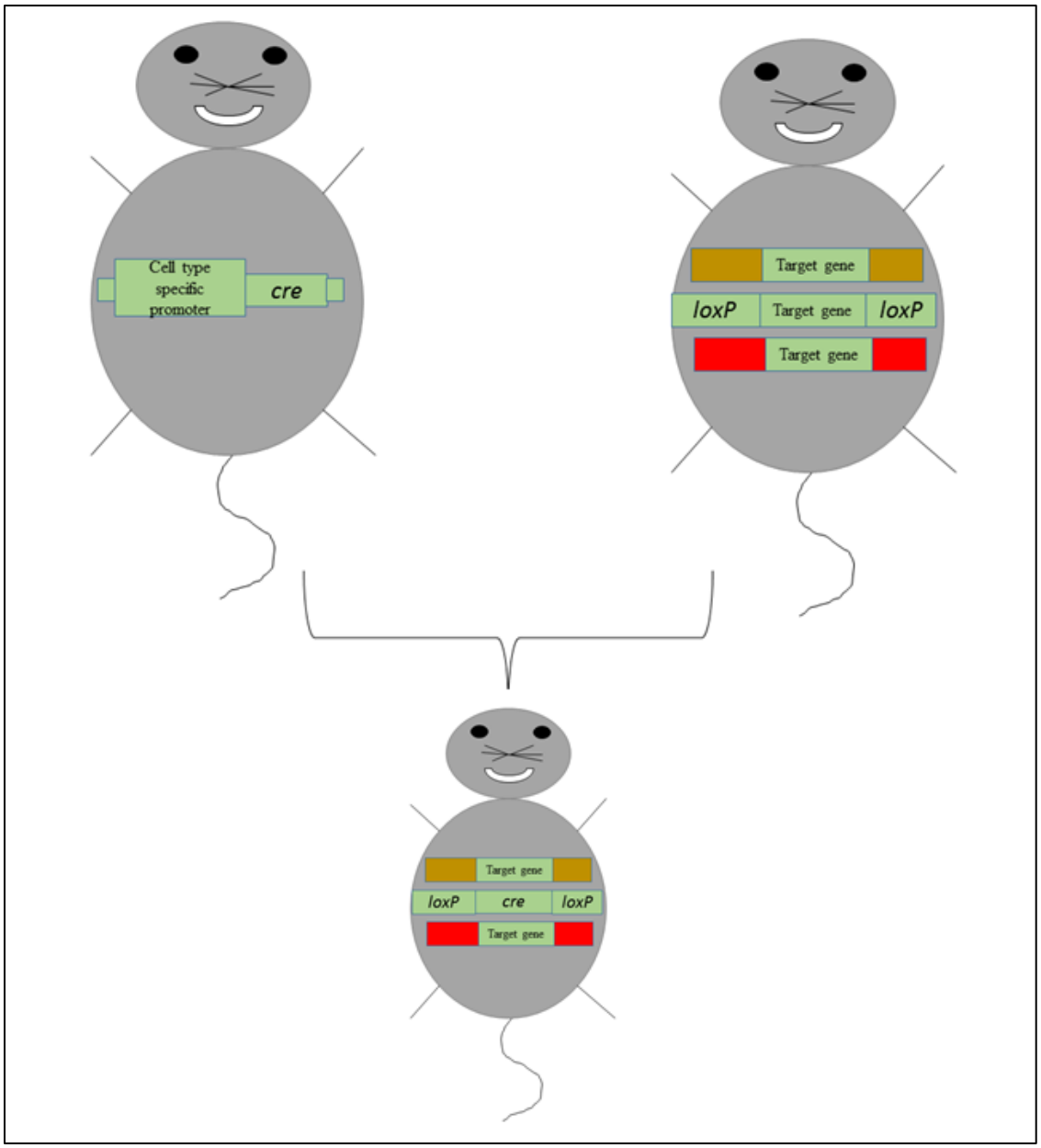


Fig. 7 The LyzMcre system for conditional gene knockout in mice.

Mice containing *loxP* flanked sequence (upper right) was mated with a mice containing cell type specific *cre* recombinase (upper left). The product of mating is a mouse (below) where *cre* deletes the floxed genomic sequences. The deletion of the genomic sequences causes a specific gene knockout of a specific cell type only. The figure has been adapted from Bouabe et al.[171].

4 AIM OF THIS WORK

Neutrophils are key effector cells of the innate immune system in pneumococcal pneumonia and there are contradictory results about the role of KLF4 in the regulation of myeloid cells function. In addition, although several reports indicate an up-regulation of KLF4 in *S. pneumoniae* infection in various cell types, however it´s *in vivo* function in pneumonia is unknown. The objective of this work is thus to evaluate the role of KLF4 in neutrophils concerning the outcome and the progression of pneumococcal pneumonia in a murine model. Therefore, the following research questions were addressed:

1. Is KLF4 expressed in neutrophils during pneumococcal pneumonia?
2. What are the virulence factors of *Streptococcus pneumoniae* that may be responsible for the expression of KLF4?
3. Does the myeloid KLF4$^{-/-}$ regulate the cytokine production of neutrophils *in vitro*?
4. Has myeloid KLF4 an impact concerning the cytokine response in the lung and in the blood of *S. pneumoniae* infected mice?
5. Is there an impact of myeloid KLF4 concerning the recruitment of cell types in the lung and blood?
6. What is the impact of myeloid KLF4 concerning the bacterial clearance in the lung, the blood and spleen?
7. Does the knockout of myeloid KLF4 alter the survival of the mice during *S. pneumoniae*-induced pneumonia and sepsis?

5 MATERIAL AND METHODS

5.1 Instruments

Table 2: *Instruments.*

Instruments	Company	Method
Autoclave	Systec	Sterilization of reagents, destruction of infected materials
Bio Photometer	Eppendorf	Bacterial growth OD values
Centrifuge	Eppendorf	Collection of supernatants (ELISA), collection of cell lysates (Western blot), preparation of bacteria
FACS Canto-II	BD	Quantification of cell populations
FilterMax F5 Multi-Mode Microplate Reader	Molecular Devices	ELISA
Incubator	Thermo Scientific	Grow and maintain cells and bacteria
Laminar Air flow bench	Thermo Scientific	Cell isolation and bacterial infection
Microscope	Olympus	Counting cells
Mini Trans-Blot® Cell	Bio-Rad	Western blot
Mini-PROTEAN® Tetra handcast system	Bio-Rad	Protein electrophoresis
Mini-PROTEAN Tetra® Vertical Electrophoresis Cell	Bio-Rad	Protein electrophoresis
Nanodrop 2000	Thermo Scientific	DNA amount and purity
Odyssey Scanner	LI-COR	Western blot membrane visualization
Photometer BioMate	Thermo Scientific	Determination of protein amount after Bradford
Polymax 1040	Heidolph Instruments	Shaking membrane (Western blot)

PTC-200 Peltier thermal cycler	MJ research	Amplification of DNA/PCR
PowerPac™ Basic power supply	Bio-Rad	Protein/DNA electrophoresis / Western blot
RCT basic	Ika-Labortechnik	Magnetic stirrer (Western blot)
Subcell® GT	Bio-Rad	DNA electrophoresis
Thermomixer Comfort	Eppendorf	Heating protein samples to 95°C (protein electrophoresis), heating DNA samples
Vet ABC ™Hematology Analyzer	Scil	Quantification of cell population

5.2 Consumable supplies

Table 3: Consumable supplies.

Supplies	Company
Cannula (diff. sizes)	Braun
Cassettes for histology	Carl Roth GmbH
Cell strainers: 70µm, 100µm	Falcon
Clear 96-well plate maxisorp	Nunc™
Columbia agar plate + 5% sheep blood	BD Mikrobiologie
Cotton stick	Applimed SA
Cuvettes	Sarstedt
K_2EDTA vials	Sarstedt
Eppendorf tubes (diff. sizes)	Sarstedt
FACS tubes	Falcon
Inoculation loop	Carl Roth GmbH
MACS pre-filters: 30µm	Miltenyi Biotec
MACS Separation columns (LS)	Miltenyi Biotec
Nitrocellulose hybond membrane	GE Healthcare Life Sciences
PCR tubes: 0.5ml	Sarstedt
Pipette tips (diff. sizes)	Biozym / Sarstedt

Seal plate	Excel Scientific Inc.
Serological pipettes (diff. sizes)	Falcon
Syringes: 1ml, 2ml, 10ml	Braun
Tubes: 15ml, 50ml	Falcon
Whatman gel blotting paper	GE Healthcare Life Sciences

5.3 Chemicals and reagents

Table 4: Chemicals and reagents.

Reagent	Company
2-propanol	Sigma-Aldrich
4% paraformaldehyde (PFA)	Carl Roth GmbH
Acrylamide / Bis-Acrylamide, 40%, Ratio 19:1	Serva
Agarose	Biozym
Albumin, from bovine serum	Sigma-Aldrich
Ammonium chloride	Sigma-Aldrich
Ammonium persulfate (APS)	Serva
Ampuwa	Fresenius Kabi
Bradford Protein Assay	Bio-Rad
Bromophenol blue	Sigma-Aldrich
Potassium hydrogen carbonate	Sigma-Aldrich
Complete™ Protease Inhibitor Cocktail Tablet	Roche
EDTA	Sigma-Aldrich
EDTA-Na salt	Carl Roth GmbH
Ethanol	Merck Millipore
Glycerol	Merck Millipore
Glycine	Merck Millipore
H_2SO_4; 95-97%	Merck Millipore
HBSS	Gibco
Heparin	Rotexmedica

Ketamin	Rotexmedica
Lysozyme	Merck Millipore
Mutanolysin	Sigma-Aldrich
N-Lauroylsarcosine sodium salt	Sigma-Aldrich
Nonidet® P-40 99%	Fluka
Nucleic acid staining solution	Red Safe™
Ody Blocking Buffer	LI-COR
PBS	Gibco
Phenol	Sigma-Aldrich
Precision plus Protein™ kaleidoscope	Bio-Rad
Proteinase K	Sigma-Aldrich
RNase	5 prime GmbH
SDS	Serva
Sodium acetate	Carl Roth GmbH
Sodium chloride	Carl Roth GmbH
Sodium fluoride 99%	Sigma-Aldrich
Sodium orthovanadat 98%	Sigma-Aldrich
Sodium pyrophosphate	Sigma-Aldrich
TEMED	Serva
TES	Sigma-Aldrich
Todd-Hewitt Broth	BD Mikrobiologie
TriDye 100bp DNA ladder	New England BioLabs
Tris base	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Xylazin	Rotexmedica

Yeast extract	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich

5.4 Antibodies

5.4.1 Antibodies for Western blot

Table 5: Antibodies for Western blot.

Antibody	Host	Type	Company
Anti-GKLF	rabbit	primary	Santa Cruz Biotechnologie
Anti-β-Actin	goat	primary	Santa Cruz Biotechnologie
Anti-rabbit conjugated Cy 5.5	goat	secondary	Rockland
Anti-goat conjugated IRDye 800	donkey	secondary	Rockland

5.4.2 Antibodies for FACS

Table 6: Antibodies for FACS.

Antibody	Company
CD11c (N418) Cy5	Biolegend
CD11b (M1/70) PE-Cy7	eBioscience™
F4/80 (BM8) PE	eBioscience™
CD45 (30-F11) FITC	BD
Ly6G (1A8) PerCP-Cy5.5	BD
Ly6C (HK1.4) BV510	Biolegend
MHC-II (M6/114.15.2) AF700	eBioscience™
Siglec F (E50-2440) BV421	BD
aCD16/32	BD

5.5 Antibiotics for selection

Table 7: Antibiotics.

Antibiotic	Company
Kanamycin	Sigma Aldrich

5.6 Stimulants

Table 8: Stimulants.

Stimulant	Origin	Usage	Company
CpG (ODN M362)	recombinant	TLR9-agonist	InvivoGen
LPS	*Salmonella Minnesota* R595	TLR4-agonist	Enzo Lifesciences
MALP-2	recombinant	TLR2-agonist	Enzo Lifesciences

5.7 Cell culture medium

Table 9: Cell culture medium.

Reagent	Company
RPMI 1640	Gibco
fetal calf serum	GE Healthcare
L-glutamine	Sigma-Aldrich

5.8 Genotyping Primers

Table 10: Genotyping primers.

Primer name	Sequence	Company
Lys olMR 3066 Mutant	5'-CCC AGA AAT GCC AGA TTA CG-3'	Metabion international AG
Lys olMR 3067 Common	5'-CTT GGG CTG CCA GAA TTT CTC-3'	Metabion international AG
Lys olMR 3068 WT	5'-TTA CAG TCG GCC AGG CTG AC-3'	Metabion international AG

KLF4 exon1	5'-CTG GGC CCA CAT TAA TGA-3'	Metabion international AG
KLF4 exon2	5'-AGT CTG ACA TGG CTG TCA GCG-3'	Metabion international AG
KLF4 intron	5'-CAG AGC CGT TCT GCC TGT TTT-3'	Metabion international AG

5.9 Kits

Table 11: Commercial kits.

Kits	Company
EasySep™ direct human neutrophil isolation kit	STEMCELL™ technologies
Mouse anti-Ly-6G Microbead kit	Miltenyi Biotec
Mouse albumin ELISA	Biomol
Mouse IL-1β ELISA	eBioscience™
Mouse TNF-α ELISA	eBioscience™
Mouse IL-10 ELISA	eBioscience™
Mouse KC ELISA	R&D Systems

5.10 Pneumococci strains

Table 12: Streptococcus pneumoniae strains.

Strain	capsule	LytA	Type of experiment
D39 wild type	yes	yes	*In vitro*
D39Δcps	no	yes	*In vitro*
R6x	no	yes	*In vitro*
R6xΔlytA	no	no	*In vitro*
NCTC 7978	yes	yes	*In vivo*

5.11 Methods

5.11.1 *In vitro* experiments

5.11.1.1 Storage and culture of *Streptococcus pneumoniae*

Different strains of pneumococci were stored at -80°C for long-term storage. Initially, the pneumococci were plated on 5% sheep blood containing Columbia blood agar plates and supplemented with antibiotics (50mg/ml kanamycin for D39Δcps and R6xΔlytA). These plates were incubated at 37°C with 5% CO_2 for 12h. After this incubation, the colonies were again transferred to new blood agar plates and incubated. Thereafter, the colonies were suspended in 1ml of cryoconservation medium (30g/l Todd-Hewitt broth with 0.5% yeast extract and 20% glycerol, pH=7.8 ± 0.2).

For infection of the cells, the bacterial strains were plated on 5% sheep blood Columbia agar plates, supplemented with the antibiotics if necessary for the mutant strains. The plates were incubated overnight at 37°C with 5% CO_2. The next day, single bacterial colonies were incubated in pre-warmed THY and allowed to grow until the OD_{600} reached 0.2-0.4 at 37°C with 5% CO_2. The bacteria containing THY was then centrifuged at 1800g for 10 minutes. The supernatant was discarded to get the bacterial pellets, which were then suspended in RPMI 1640 with 2% FCS and 1% glutamine to reach a concentration of $1x10^9$ CFU/ml. This concentration was further diluted via serial dilution to get the required concentration for the infection of cells.

5.11.1.2 Isolation of R6x DNA

R6x strain was plated on Columbia blood agar plates and incubated overnight at 37°C, 5% CO_2. The next day, 2-3 colonies were incubated in pre-warmed THY and the bacteria was allowed to grow until the OD_{600} value reached 0.6. The THY containing bacteria was then centrifuged at 1800g for 10 minutes at 4°C. The bacterial pellets were resuspended in 6ml TES. For lysis of the cells, 1ml of lysozyme (5mg/ml in TES) was added followed by addition of 100µl of Mutanolysin (1mg/ml in Tris-HCl pH 7.5). This mixture was incubated at 37°C for 1h. 100µl of RNase (5mg/ml in TES with 15 minutes pretreatment at 75°C to inactivate DNase) was added and again incubated at 37°C for 15 minutes. 100µl Proteinase K was added to it and kept at 30°C for 30 minutes. This was followed by further addition of 500µl 10% N-Lauroylsarcosine sodium salt (in 250mM EDTA) and allowed to react for 1h at 37°C. 2ml phenol was added followed by shaking and centrifugation at 3023g for 10 minutes. The supernatant was collected and precipitated with 1/10vol 3M sodium acetate and 1vol 2-

propanol. Pellets of DNA will be seen separating out. If pellets of DNA were not observed, 2-propanol was further added. The DNA pellets were suspended in 1ml 70% ethanol and centrifuged at 4487g for 5 minutes. After centrifugation, the DNA pellets were suspended in Tris-EDTA buffer (1M Tris-HCl, 0.5M EDTA, pH 8.0) and the concentration was measured using Nanodrop.

5.11.1.3 Isolation of neutrophils from human blood

Blood was taken from healthy subjects with their prior consent. Around 10ml was collected in K_2EDTA tubes for each set of experiments. Neutrophils were then isolated by negative selection using the EasySep™ (STEMCELL™ technologies). The cells were then resuspended in RPMI 1640 with 2% FCS and 1% glutamine before stimulation with bacteria, bacterial DNA or TLR agonist. Cells numbers were determined by Neubauer chamber.

5.11.1.4 Isolation of neutrophils from mouse bone marrow and whole blood

Animal experiments were done based on FELASA guidelines. All *in vitro* experiments using cells of mice were approved by the host institution (Charité - Universitätmedizin Berlin) and governmental (LAGeSo Berlin, registration no.: T0087/15). All experiments were performed under *i.p.* anesthesia of ketamine and xylazine followed by cervical dislocation or exsanguinations *via* the *vena cava caudalis*.

For the isolation of neutrophils from bone marrow, the extremities were removed from the mice. Subsequently, the connecting tissues attached to the bones were removed. The bones were sterilized in 70% ethanol for 5 minutes and then immersed in RPMI 1640. The bones were crushed carefully using a mortar without hurting the cells. The RPMI 1640 containing cell suspension was passed through a 70µm cell strainer followed by a centrifugation at 300g for 10 minutes at 4°C. After centrifugation at 300g for 10 minutes at 4°C, the cells were resuspended in 1-2ml MACS buffer (0.5% BSA in 2mM EDTA dissolved PBS, pH 7.2) and passed through a 30µm MACS pre-filter. The neutrophils were then isolated using the MACS mouse anti-Ly-6G kit by means of positive selection. The isolated neutrophils, after determining the cell number in microscope, was re-suspended in RPMI 1640 with 2% FCS, 1% glutamine and used for experiment immediately. Cell numbers were determined by Neubauer chamber.

For isolation of neutrophils from whole blood, blood was collected from the *vena cava caudalis* after application of 50µl of heparin into it. Blood from at least 10 mice in each group was pulled down to get enough cells for one independent experiment. After the blood was

collected in 50ml tubes, red blood cells were lysed using red blood cell (RBC) lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 10nM EDTA-Na; pH 7.4). The RBC lysis was repeated again to get rid of all the red blood cells and then the neutrophils were isolated using the MACS mouse anti-Ly-6G kit by means of positive selection. The other fraction of the cells (except for the RBC) was also collected for further analysis. The cells were resuspended with in RPMI 1640 supplemented with 2% FCS and 1% glutamine. Cell numbers were determined by Neubauer chamber.

5.11.1.5 Genotyping and PCR

For genotyping of mice, ear biopsies were obtained from the mice. These biopsies were incubated overnight in lysis buffer (1M Tris-HCl, 0.5M EDTA, 5M NaCl, 10% SDS in distilled water) with Proteinase K (10mg/ml). The incubation was done in thermo-block at 54°C. Next day, the tubes were flipped upside down to mix properly and then centrifuged at 3824g for 10 minutes. After centrifugation, the supernatant was collected and 500µl 2-propanol was added, mixing the component well. Subsequently, a centrifugation at 15294g for 5 minutes was done with this mixture. Following the removal of the supernatant, 500µl 70% ethanol was added to wash the DNA pellet. After adding ethanol, it was again centrifuged at 17949g for 5 minutes and the supernatant was carefully discarded. The DNA pellets were air dried and dissolved in 10µl nuclease free water. The concentration of the DNA was measured thereafter using a Nanodrop.

For the PCR amplification of LyzMcre, 50ng DNA was added to components as given in table 13. For each DNA sample, a pair of PCR mixture was prepared, namely-mutant and wildtype.

Table 13: Components of the PCR mixture for LyzMcre.

Mutant		**Wildtype**	
Component	**Volume**	**Component**	**Volume**
10x DreamTaq green buffer	5µl	10x DreamTaq green buffer	5µl
dNTPs	1µl	dNTPs	1µl
DreamTaq DNA polymerase	0.5µl	DreamTaq DNA polymerase	0.5µl

Primer Lys olMR 3066 Mutant	0.3µl	Primer Lys olMR 3068 WT	0.3µl
Primer Lys olMR 3067 Common	0.3µl	Primer Lys olMR 3067 Common	0.3µl
Water	37.9µl	Water	37.9µl
DNA	5µl	DNA	5µl
Total Volume	50µl	Total Volume	50µl

The PCR cycle was also different for the two sets and is given in table 14 and 15. PCR was performed in PTC-200 Peltier thermal cycler.

Table 14: PCR cycle for mutant probes.

Cycle name	**Temperature of the cycle (°C)**	**Time of each cycle**	**Total number of cycles**
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	40
Annealing	62	60 seconds	40
Extension	72	60 seconds	40
Final extension	72	2 minutes	40

Table 15: PCR cycle for wildtype probes.

Cycle name	**Temperature of the cycle (°C)**	**Time of each cycle**	**Total number of cycles**
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	40
Annealing	67	60 seconds	40
Extension	72	60 seconds	40

Final extension	72	2 minutes	40

The same isolated DNA was also used for flox amplification. For the flox amplification, 50ng DNA was mixed with the following components as given in table 16.

Table 16: Components of PCR mixture for flox.

Components	Volume
10x DreamTaq green buffer	5µl
$MgCl_2$	2µl
dNTPs	1µl
DreamTaq DNA polymerase	0.5µl
Primer exon1	0.3µl
Primer exon2	0.3µl
Primer intron	0.3µl
Water	35.6µl
DNA	5µl
Total volume	50µl

PCR amplification for flox was done in PTC-200 Peltier thermal cycler and the cycle conditions are given in table 17.

Table 17: PCR cycle conditions for flox amplification.

Cycle name	Temperature of the cycle (°C)	Time of each cycle	Total number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	32
Annealing	60	30 seconds	32

Extension	72	60 seconds	32
Final extension	72	7 minutes	32

For the gel electrophoresis, 20µl PCR samples were run on 1% agarose gel with 0.04% Red Safe™ nucleic staining solution for 45 minutes at 70 volts. For the LyzMcre genotyping, the wildtype PCR probe and mutant PCR probe were loaded in adjacent pockets for the respective mice. Along with the samples, a reference DNA ladder (TriDye 100bp DNA ladder, New England BioLabs) was also loaded. The bands were visualized with ultraviolet light and documented with a camera. The description of the flox genotyping for the representative samples (1-6) in Fig. 8A are given in table 18. The description of the LyzMcre genotyping for the representative samples (1-7) in Fig. 8B are given in table 19.

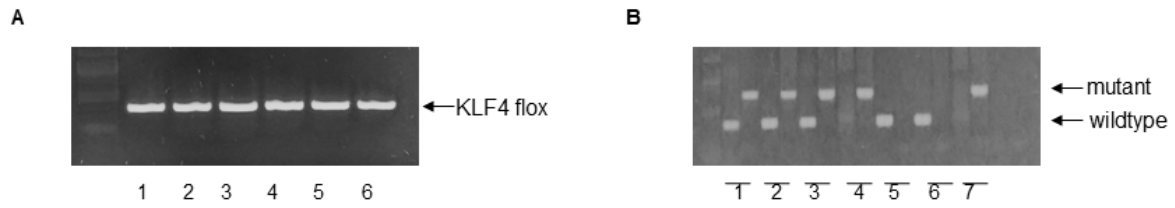


Fig. 8 Genotyping of mice for in vitro and in vivo experiments.

Ear biopsies of mice were used for DNA extraction and in turn for flox and LyzMcre genotyping. All the mice used in our study are flox++ so only band ~300 bp long was visible as given in the representative gel picture (Fig. 8A). For the LyzMcre genotyping, each mice have a pair of PCR samples, known as wildtype and mutant. The length of wildtype band is ~350bp and the mutant band is ~700 bp as given in the representative gel picture (Fig. 8B).

Table 18: Evaluation for flox++.

Sample No.	KLF4 flox (~300bp)	Mice type
1	yes	Flox++
2	yes	Flox++
3	yes	Flox++
4	yes	Flox++
5	yes	Flox++

6	yes	Flox++

Table 19: Screening of mice for LyzMcre.

Sample No.	Wildtype (~350bp)	Mutant(~700 bp)	Mice type
1	yes	yes	KLF4 heterozygote or $KLF^{+/-}$
2	yes	yes	$KLF^{+/-}$
3	yes	yes	$KLF^{+/-}$
4	no	yes	$KLF4^{-/-}$
5	yes	no	$KLF4^{+/+}$
6	yes	no	$KLF4^{+/+}$
7	no	yes	$KLF4^{-/-}$

5.11.1.6 Infection of cells

Isolated neutrophils from human blood or mouse bone marrow/blood were always stimulated in 1.5-2ml tubes. To infect the cells, RPMI 1640 medium containing 2% FCS and 1% glutamine with bacterial suspension was added to cells ($1x10^6$ CFU/ml – $1x10^8$ CFU/ml for MOI1-100 respectively). For the control cells, only RPMI 1640 medium with 2% FCS and 1% glutamine was added. For the stimulation with R6x DNA, the concentration was 5µg/ml. For the stimulation with the TLR agonists, MALP-2 was used at a concentration of 0.05ng/µl, LPS at a concentration of 0.1ng/µl and CpG at a concentration of 10ng/µl.

For the Western blot experiments, the stimulation was done for 3h and 6h. For the ELISA experiments, the stimulation was done for 16h.

5.11.1.7 Phagocytosis assay by neutrophils

Streptococcus pneumoniae strains were grown in THY and resuspended in HBSS with Ca^{2+} and Mg^{2+} at a concentration of $1x10^8$ CFU/ml. 10µl of this bacterial suspension (equivalent to $1x10^6$ CFU/ml) was taken with 40µl mouse serum of the respective strain and opsonized for 30 minutes at 37°C. To this 100µl of RPMI 1640 containing neutrophils isolated from mice blood (concentration of cells $1x10^4$) was added. Thereafter, it was incubated at 37°C for 1h.

After this, 50µl of lysis buffer (20µl of 10% Triton X-100 + 30µl HBSS with Ca^{2+} and Mg^{2+}) was added and kept for 10 minutes at 37°C. This was plated in serial dilutions in Columbia blood agar plates and viable bacterial colonies were counted the next day.

5.11.1.8 Western blot

5.11.1.8.1 Extraction of protein

At the respective time points after the infection, the tubes were centrifuged at 1700g for 3 minutes at 4°C. The supernatants were discarded and 40µl of lysis buffer was added to pellet. Thereafter it was stored at -20°C overnight. The composition of the lysis buffer is given in table 20. The next day, the tubes were thawed on ice and centrifuged at 15294g for 10 minutes at 4°C. The supernatants were collected and transferred to new tubes and kept at -20°C for storage.

Table 20: Composition of lysis buffer for Western blot.

Buffer	Composition
Phosphoprotein wash buffer	5ml sodiumorthovanadat 98% (Stock solution 200mM) 50ml sodium pyrophosphate (Stock solution 150mM) 50ml sodium fluoride 99% (Stock solution 1M) 395ml distilled water
Phosphoprotein lysis buffer	100µl Tris-HCl, pH 7.4 (Stock solution 500mM) 50µl (v/v) Nonidet® P-40 (Stock solution 20%) 40µl Complete™ Protease Inhibitor Cocktail 810µl phosphoprotein wash buffer

5.11.1.8.2 Estimation of total protein

Total protein content in the samples were measured using the Bio-Rad protein assay following the manufacturer's protocol. The OD_{595} was measured using spectrophotometer.

5.11.1.8.3 Preparation of samples for electrophoresis

Laemmli buffer was added to the samples in the ratio of 1:4.The samples were heated to 95°C for 5 minutes in thermo-block. The samples were cooled on ice before loading them into the gel for electrophoresis. The composition of the laemmli buffer is given in table 21.

Table 21: Composition of the Laemmli buffer.

Buffer	Composition
Laemmli buffer	1ml Tris-HCl, pH 6.8 (500mM) 0.8ml glycerol 1.6ml SDS 0.4ml bromophenolblue 0.4ml β-mercaptoethanol 5.0ml distilled water

5.11.1.8.4 SDS-PAGE and immunoblot

In order to resolve the protein in the samples based on their size, the samples were run on sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). 40µg of protein was loaded into each pocket of 10% SDS-PAGE, which is composed of the upper stacking and lower resolving gel. The electrophoresis for separation was done at 100 volts (constant voltage) for 1.5-2h in running buffer. Along with the samples was also loaded a reference protein marker (Precision plus Protein™ kaleidoscope, Bio-Rad). The composition of stacking gel, resolving gel and running buffer is given in table 22.

For Western blot, the proteins were wet transferred to a nitrocellulose membrane at 100 volts (constant voltage), for 1h under cool conditions. The membranes were incubated with Ody blocking buffer for 2h at room temperature to prevent nonspecific binding of the antibodies to other proteins. The membranes were incubated overnight with anti-GKLF antibody at 4°C with shaking so that the antibody spread uniformly throughout the membrane. The next day, the membranes were washed 2 times with PBST (1x PBS + 0.01% Tween-20) followed by 1

time wash with PBS. The membranes were incubated with the secondary fluorescent conjugated antibody for 1h at room temperature. The membrane was again subjected to similar washing-2 times with PBST (1x PBS+0.01% Tween-20) followed by once wash with PBS. The membranes were scanned using odyssey scanner and the KLF4 band was quantified using Image studio 5.2.

After scanning, the membrane was incubated with anti-β-Actin antibody for 1h at room temperature to check the loading control. Following a similar wash with PBST and PBS, the membranes were incubated with secondary fluorescent conjugated antibody for 30 minutes at room temperature. The membranes were finally washed with PBST and PBS like before, scanned and quantified for Actin. The composition of the transfer buffer is given in table 22 and the antibody dilutions are given in table 23.

Table 22: Composition of buffers for SDS-PAGE and immunoblotting.

Buffer/Solution	**Composition**
Resolving gel (2x)	4.85ml distilled water 2.5ml Tris-HCl pH 8.8 (stock Solution 500mM) 2.5ml Acrylamide/Bis-Acrylamide (40%, ratio 19:1) 100µl 10% SDS 50µl 10% APS 5µl TEMED
Stacking gel(2x)	2.426ml distilled water 1.0ml Tris-HCl pH 6.8 (stock Solution 1.5M) 0.534ml Acrylamide/Bis-Acrylamide (40%, ratio 19:1) 40µl 10% SDS 40µl 10% APS 10µl TEMED

Running Buffer	3g Tris base 14.4g glycine 1g SDS 1000ml distilled water
Transfer buffer	3g Tris base 14.4g glycine 20% methanol 1000ml distilled water

Table 23: Dilutions of the antibodies used for Western blot.

Antibody	Dilution in odyssey blocking buffer
Anti-GKLF	1:1000
Anti-β-Actin	1:1000
Anti-rabbit conjugated Cy 5.5	1:2000
Anti-goat conjugated IRDye 800	1:2000

5.11.1.9 Enzyme-linked Immunosorbent Assay (ELISA)

For the ELISA, the stimulation was done for 16h. At the end of the time point of infection, the samples were centrifuged at 300g for 10 minutes at 4°C. The supernatants were collected and kept at -20°C for storage.

All ELISAs were performed according the manufacturer's instruction (eBioscience™ or R&D systems). Maxisorp 96 well plates were used for the analysis and the optical density was measured at 450 nm using multi-mode microplate reader (FilterMax F5). A standard curve was generated and the results were expressed in pg/ml.

5.11.2 *In vivo* experiments

The local instituition (Charité - Universitätmedizin Berlin) and governmental organization (LAGeSo Berlin, registration no.: G0028/16) approved all the *in vivo* experiments. Female

mice with C57BL/6 background, aged 8-11 weeks were kept in individually ventilated cages and were provide food and water *ad libitum*. All experiments were performed under *i.p.* anesthesia of ketamine and xylazine.

C57BL/6 ERT-Cre$^{+/-}$/Klf4loxP/loxP mice and C57BL/6 ERT-Cre$^{-/-}$/Klf4loxP/loxP mice (kind gift from Gary K. Owen, University of Virginia, Charlottesville) were mated with B6.129P2-Lyz2tm1(cre) Ifo mice (Charles River, Sulzfeld, Germany) to generate the KLF4 WT mice or KLF4$^{+/+}$ (C57BL/6 LyzMCre$^{-/-}$/Klf4$^{loxP/loxP}$) and KLF4 KO mice or KLF4$^{-/-}$ (LyzMcre$^{+/+}$/Klf4$^{loxP/loxP}$).

5.11.2.1 Mouse pneumonia model

S. pneumoniae (NCTC 7978) was grown in THY. The bacteria were resuspended in PBS before infecting the mice. Mice were anesthetized and transnasal infection of 5x10^5 CFU/ml was done for the CFU, ELISA, FACS, histology experiments. A dosage of 5x10^4 CFU/ml was done for the survival experiment as the experiment would continue for 10 days after the infection. For both the KLF4$^{-/-}$ and KLF4$^{+/+}$ mice groups, the control mice were given transnasal dosage of PBS. The animals were kept in 12h light/dark cycle and also monitored every 12h for clinical symptoms (including temperature and weight) until the end of the experiment. The animals were euthanized when their level of pain reached the prescribed human end point.

5.11.2.2 CFU measurement in the lung, blood and spleen of infected mice

KLF4$^{-/-}$ and KLF4$^{+/+}$ mice were sacrificed 24h after infection. The blood was collected from the *vena cava caudalis* and kept in K_2EDTA tubes to prevent clotting. The lung and the spleen samples were removed from the mice, homogenized in chilled PBS and passed through a 100µm cell strainer. Serial dilution of the blood, lung and spleen homogenates were made with PBST. The samples were plated on 5% sheep blood Columbia blood agar plates and incubated at 37°C, 5% CO_2 overnight. The colonies were counted the next day.

5.11.2.3 Preparation of Broncho-alveolar lavage fluid (BALF) and plasma

KLF4$^{-/-}$ and KLF4$^{+/+}$ mice were anesthetized, 24h after infection. Blood was collected from the *vena cava caudalis* and stored in K_2EDTA tubes at 4°C. The tubes were then centrifuged

at 2000g for 10 minutes at 4°C and the supernatant (plasma) was collected and transferred to new 1.5ml tubes. For the preparation of BALF, a cannula was inserted into the trachea of the mice and the lungs were flushed twice with 800µl of chilled PBS for the collection of BALF.

5.11.2.4 Analysis of cell recruitment in blood and BALF

Blood was collected from the mice and stored in K_2EDTA tubes. The cell population was estimated in whole uncoagulated blood using the Scil Vet ABC ™Hematology Analyzer. BALF was collected and staining with required antibodies were done. Surface block (given in table 24) was added to 100µl of BALF sample in FACS tubes and incubated on ice for 5 minutes. Thereafter, the surface staining antibody mixture (given in table 25) was added to it and incubated for 30 minutes on ice in dark. Subsequently, the FACS tubes were filled up with PBS, centrifuged at 425g for 5 minutes at 4°C and the supernatant was discarded. 180µl of 1% Paraformaldehyde (PFA) in PBS was added to fix the cells and incubated overnight at 4°C. The next day, the tubes were filled with FACS buffer (0.2% BSA in PBS) and centrifuged at 425g for 5 minutes at 4°C. After discarding the supernatant, the samples were resuspended in 100µl FACS buffer and measured in BD Canto-II. The cell populations were quantified were using FlowJo version 10.4.2.

Table 24: Composition of the surface block.

Antibody	Amount in 100µl staining volume (µl)
aCD16/32	0.5
FACS buffer	9.5
Total volume	10

Table 25: Composition of the surface staining antibodies.

Antibody (clone)	colour	Amount in 100µl staining volume (µl)
CD11c (N418)	Cy5	0.4
CD11b (M1/70)	PE-Cy7	0.5
F4/80 (BM8)	PE	0.8
CD45 (30-F11)	FITC	0.25
Ly6G (1A8)	PerCP-Cy5.5	0.5
Ly6C (HK1.4)	BV510	0.5

MHC-II (M6/114.15.2)	AF700	0.15
Siglec F (E50-2440)	BV421	0.8
FACS Buffer	---	16.1
Total volume	---	20

5.11.2.5 Mouse albumin ELISA

Mouse plasma and BALF was collected as described previously. Samples were diluted as given in table 26. Mouse albumin was measured using mouse albumin ELISA kit (BioMol, Hamburg, Germany). Different dilutions have been used for different sample types so that the OD values are within the detection range of the ELISA kit. The OD values were multiplied by their respective dilution factors to get the actual albumin content in the BALF and plasma. For the quantification of vascular permeability, the albumin content in the BALF was divided by the albumin content in the plasma of the respective mouse and multiplied by 1000 to get values greater than 0.00.

Table 26: Dilution of samples for mouse albumin ELISA.

Sample	Type of infection	Dilution
BALF	PBS	1:1000
BALF	*S.p.*	1:10000
Plasma	PBS	1:500000
Plasma	*S.p.*	1:500000

5.11.2.6 Preparation of lungs and other organs for histological analysis

This part of the study was done in collaboration with Prof. Achim Gruber (Department of veterinary pathology, Freie Universität Berlin). The mice were anesthetized 24h post infection and the whole lungs were removed carefully along with the trachea and put in the embedding cassette (Carl Roth GmbH). Similarly organs such as thymus, heart, spleen, liver, small intestine, large intestine, kidneys were also removed and put in embedding cassettes. The brain was also removed and put in 50ml tube. All the samples were fixed in 4% PFA pH=7.0 up to 48h and embedded in paraffin. Multiple sections were cut from the paraffin embedded samples and analyzed for histopathology markers[172,173,174]. Additionally lung samples were also stained for *S. pneumoniae* antibody (as kind gift from Prof. S. Hammerschmidt, Ernst-

Moritz-Arndt Universität Greiswald) to ascertain the bacterial load in the lung as have been previously described[175].

5.11.3 Statistical Analysis

Statistical analysis was done using GraphPad Prism 6. Mann-Whitney U test was used for comparison of two populations. For the analysis of cytokines *in vitro*, unpaired t-test was used. For comparison of populations for more than two groups, one way ANOVA followed by Dunn's multiple comparison test was used. For the survival experiment, Mantel-Cox log rank test was performed. Multiple t-test was performed for comparison of the weight and temperature between the two groups of the survival experiment.

6 RESULTS

6.1 *In vitro*

6.1.1 *S. pneumoniae* induces KLF4 expression in human neutrophils in a time and dose dependent manner but this expression is not dependent on TLR2, 4, 9.

Since we have already seen an increased expression of KLF4 in BMMs during pneumococcal pneumonia from previous studies, which had a functional role[169], experiments were done to see whether neutrophils showed any KLF4 expression during bacterial pneumonia. Human neutrophils were isolated from whole blood obtained from healthy individuals. The cells were stimulated for 3h (Fig. 9A) and 6h (Fig. 9B-D) with different doses (Multiplicity of Infection (MOI) 1, Fig. 9A and B or MOI10, Fig. 9C-D) of *Streptococcus pneumoniae*. The samples were lysed for Western blot analysis for checking the protein expression of KLF4. Unstimulated neutrophils (C) did not show any KLF4 expression (Fig. 9A-D). It was observed that there was a strong induction of KLF4 in human neutrophils in case of the unencapsulated *S. pneumoniae* strain (R6x). There was significant induction of KLF4 at MOI1, both after 3h and 6h stimulation. (Fig. 9A and B respectively). For the wildtype *S. pneumoniae* strain (D39) and the other unencapsulated *S. pneumoniae* strain (D39Δcps), higher MOIs were needed for KLF4 expression (Fig. 9C and D respectively). Neutrophils isolated from human blood were also stimulated with TLR ligands. MALP-2 (0.05ng/µl) was used as TLR2 ligand, CpG (10ng/µl) as TLR9 agonist while LPS (0.1ng/µl) was used as TLR4 ligand for 6h. No induction of KLF4 was seen in human neutrophils after stimulation with the TLR-ligands as given in Fig. 9B.

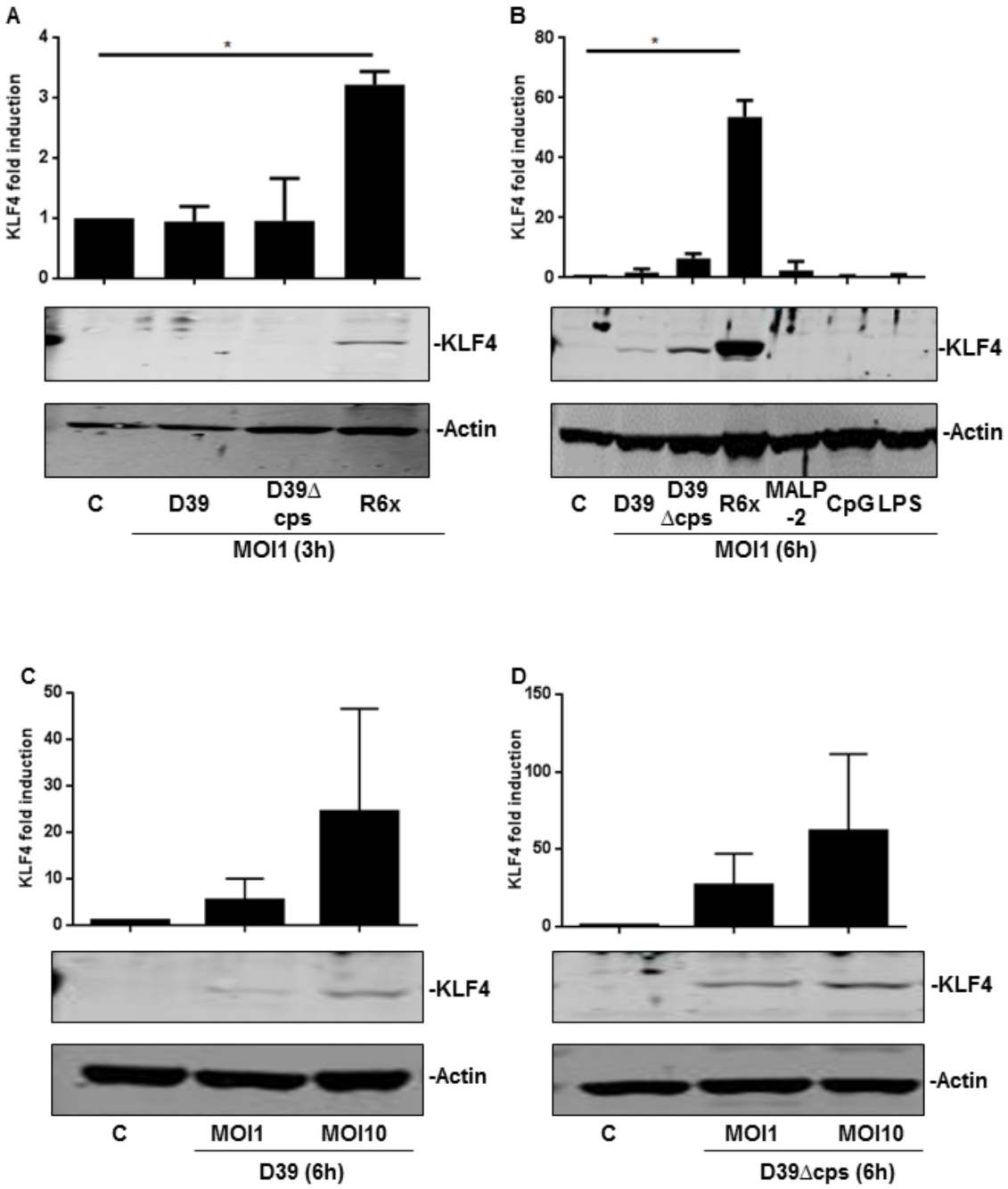


Fig. 9 KLF4 is induced in human neutrophils by S. pneumoniae.

Neutrophils were stimulated for 3h (Fig. 9A) or 6h (Fig. 9B-D) with *S. pneumoniae* strains R6x (Fig. 9A and B), D39 (Fig. 9A-C) and D39Δcps (Fig. 9A, B and D) with different MOIs. Neutrophils were also stimulated with TLR2, 4, 9 ligands for 6h (Fig. 9B). The cell lysates were used for Western blot analysis. The membranes were incubated with primary antibody overnight (KLF4) as given in the upper panel in Fig. 9A-D. Actin was detected on the same membrane as the loading control (lower panel in Fig. 9A-D). The membranes were scanned using LI-COR Odyssey scanner and quantification of the membranes (band intensity) was done using image studio ver2.0. Fold induction was calculated by the ratio of KLF4 and Actin. The blots shown are representatives from three independent experiments. The graphs are shown as Mean ± SD and the results were analyzed with one-way

ANOVA followed by Dunn's multiple comparison test. Statistically significant differences were characterized using graphpad prism software, by asterisks as follows: P<0.05 *.

6.1.2 Pneumococci-induced expression of KLF4 depends on autolysis

Zahlten et al. and Herta et al. have previously shown that LytA (autolysin) dependent release of bacterial DNA was a controlling factor in the induction of KLF4 in epithelial cells and macrophages during *S. pneumoniae* infection[167,169]. The present experiment was done to see whether the induction of KLF4 was also dependent on autolysin like in the other cell types. Human neutrophils (Fig. 10A) and neutrophils isolated from the Bone Marrow (BM) of WT C57BL/6 mice (Fig. 10B), were stimulated with R6x, the autolysin deficient R6x (R6xΔlytA), R6x DNA, and combination of R6xΔlytA and R6x DNA. The stimulation was done for 6h. The whole cell lysates were the used for Western blot analysis. The induction of KLF4 due to R6x was reduced in the R6xΔlytA. DNA alone was not sufficient to induce KLF4 but the KLF4 signal could be partly brought back only when the neutrophils were stimulated with the combination of R6xΔlytA and R6x DNA.

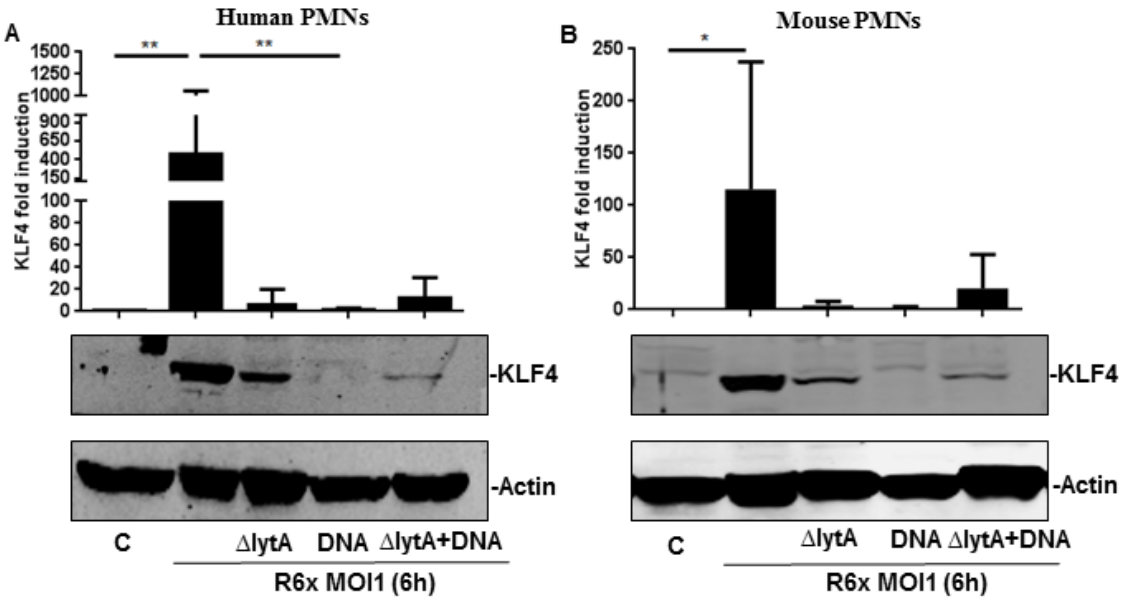


Fig. 10 The expression of KLF4 in human and mice neutrophils due to pneumococci depends on autolysis.

The cell lysates after 6h stimulation was used for Western blot and the membranes were incubated with KLF4 and Actin antibody sequentially (Fig. 9). It was observed that the induction of KLF4 due to R6x in human neutrophils (Fig. 10A) and mice BM-derived neutrophils (Fig. 10B) was reduced and not induced when the cells were stimulated with R6xΔlytA and R6x DNA respectively. However, the reduced induction of KLF4 could be partly reversed when the combination of R6xΔlytA and R6x DNA was used for stimulation. In all stimulations, a MOI1 was used. The control cells (C) are the cells without any stimulation (Fig. 10A and B). The upper panel is KLF4 and lower panel is Actin, which is used as the loading control. Blots shown are representatives from three-six independent experiments in Fig. 10A and from four independent experiments in Fig. 10B. The graphs are

shown as Mean ± SD and the results were analyzed with one-way ANOVA followed by Dunn's multiple comparison test. Statistically significant differences were characterized using graphpad prism software, by asterisks as follows: P<0.05 * and P< 0.01 **.

6.1.3 The LyzMcre system has a functional KO in blood derived neutrophils but not in BM derived neutrophils

After the initial screening of mice with genotyping PCR, BM-derived neutrophils and blood derived neutrophils were isolated from KLF4$^{+/+}$ (Fig. 11A, B), KLF4$^{-/-}$ (Fig. 11A, B) and also KLF4$^{+/-}$ (only Fig. 11A). The cells were then stimulated for 6h (MOI1) with R6x and the cell lysates were used for Western blot analysis. There was hardly any KO in the BM-derived neutrophils (Fig. 11A) but a significant knockdown (~90%) of KLF4 in neutrophils isolated from blood but not in the other white blood cells (Fig. 11B) as seen in the Western blot data.

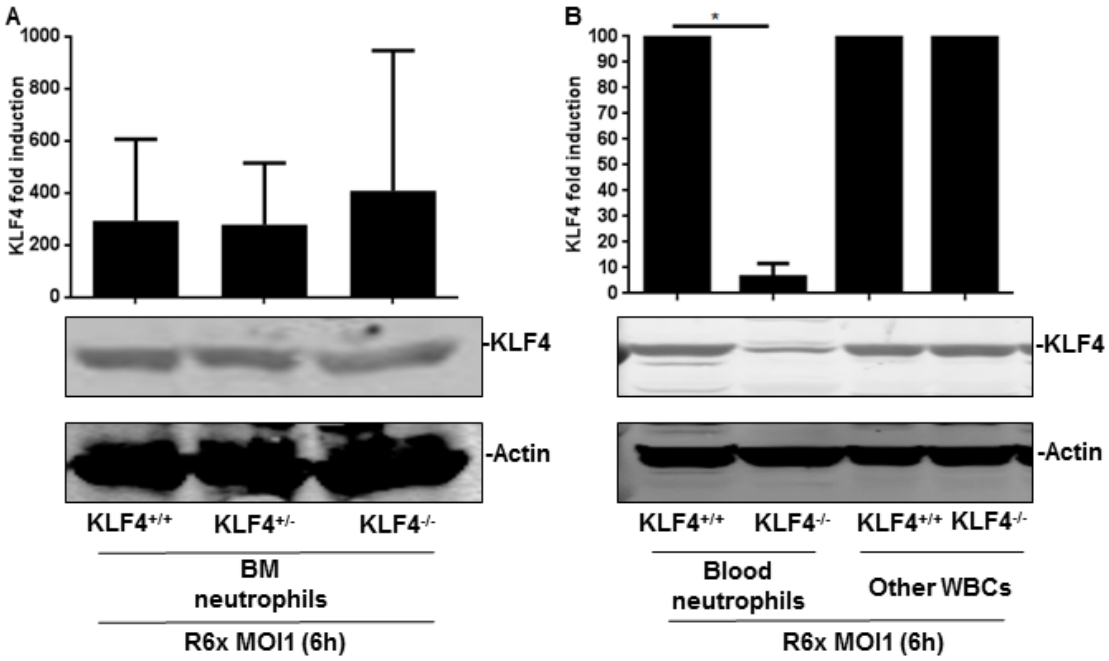


Fig. 11 LyzMcre causes a KLF4 KO only in blood derived neutrophils.

The cell lysates were used for Western blot and incubated with KLF4 antibody, as given in the upper panel. Actin used as a loading control is given in the lower panel. Blots shown are representative from three independent experiments in neutrophils isolated from the BM and blood (Fig. 11A and B). The graphs are shown as Mean ± SD and results were analyzed using one way-ANOVA followed by Dunn's multiple comparison test and P<0.05 * was defined as the level of significance.

6.1.4 KLF4$^{-/-}$ and KLF4$^{+/+}$ neutrophils do not show any differences in bacterial killing *in vitro*

Phagocytosis and killing of bacteria are central important functions of myeloid cells to clear bacterial infections. Therefore, it was tested whether KLF4$^{-/-}$ did have any impact in the

bacterial killing by neutrophils. Neutrophils isolated from blood of KLF4$^{+/+}$ and KLF4$^{-/-}$ mice were stimulated with *S. pneumoniae* D39 and R6x for 1h (Fig. 12A and B). In both cases, a MOI100 infection was tested. The cells were then plated on blood agar plates in serial dilutions, and CFU were counted the next day. However, the neutrophils show their inherent bacterial killing activity (~ 1.5 log scales) but there were no differences in bacterial killing by neutrophils with respect to KLF4$^{-/-}$.

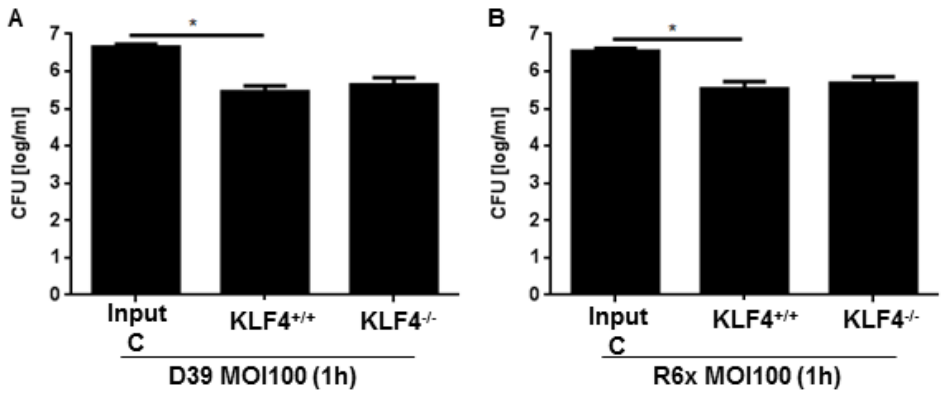


Fig. 12 Analysis of the bacterial killing in KLF4$^{-/-}$ neutrophils.

Fig. 12A and B represent the CFU results of neutrophils stimulated with D39 (A) and R6x (B) respectively from three independent experiments. Input C (Input control) are the bacterial dilutions without the neutrophils. The graphs are shown as Mean ± SD and the results were analyzed with one-way ANOVA followed by Dunn's multiple comparison test. Statistically significant differences were characterized using graphpad prism software, by asterisks as follows: P<0.05 *.

6.1.5 KLF4$^{-/-}$ neutrophils produce less TNF-α and KC

Besides their direct killing activity of neutrophils, these cells contribute to the regulation of the innate immune response by the liberation of inflammatory mediators[119,120]. Therefore, the effect of KLF4 protein presence or absence on the pneumococci-related liberation of such mediators was tested. 16h after stimulation with *S. pneumoniae* D39, neutrophils with KLF4$^{-/-}$ produce significantly less TNF-α (Fig. 13A) and significantly less KC (Fig. 13B). Noteworthy, there was no KC measurable in the KLF4$^{-/-}$ neutrophils (under detection limit). Since it is now known that there is no knockdown of KLF4 in the other white blood cells (see Fig. 11B) these cells were used as a control. Fig. 13C-D shows that there were no differences in the cytokine response to *S. pneumoniae* in the WBCs (excluding neutrophils) indicating a specific effect of the KLF4$^{-/-}$ in neutrophils concerning the cytokine response.

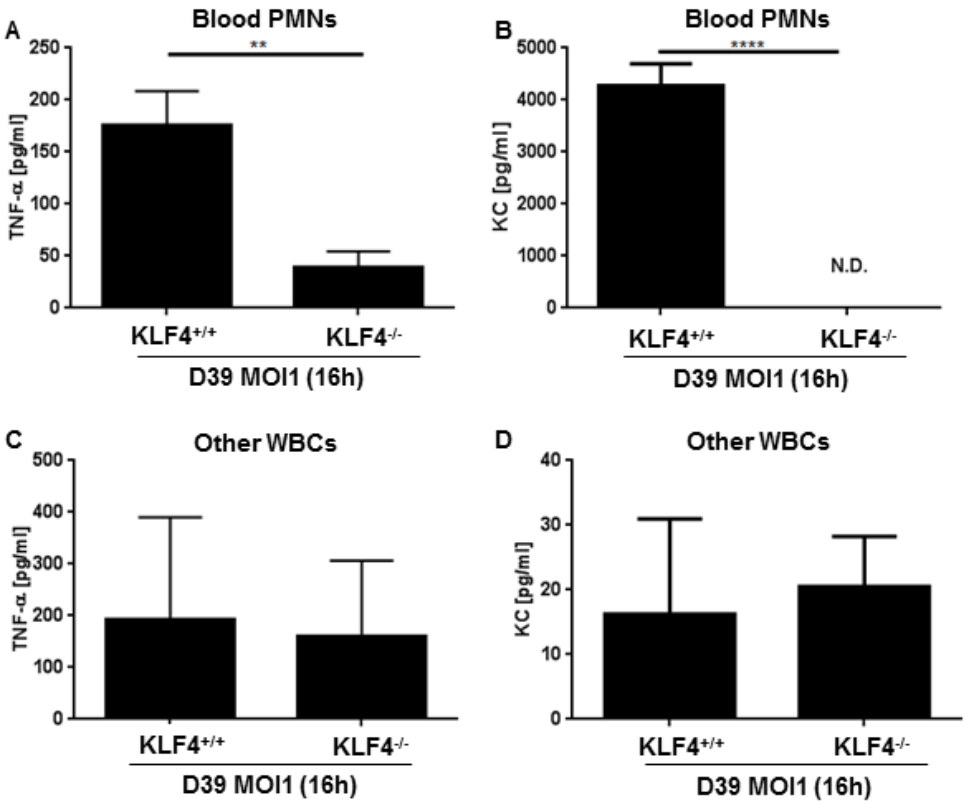


Fig. 13 KLF4$^{-/-}$ specific alteration of pneumococci induced cytokines in neutrophils.

The cells after isolation were stimulated with *S. pneumoniae* D39 for 16h (MOI1). The supernatants were used for cytokine measurement. The various cytokine/chemokines levels were measured using commercially available ELISA kits following the manufacturer's instruction. For neutrophils: TNF-α and KC in Fig. 13A and B respectively and for WBCs: TNF-α and KC in Fig. 13C and D respectively. The graphs are shown as Mean ± SD from three independent experiments and the results were analyzed using unpaired t-test. Statistically significant differences were characterized using graphpad prism software, by stars as follows: P< 0.01 **; P<0.0001 ****. N.D. = not detectable.

6.2 *In vivo*

6.2.1 Higher bacterial load in lungs, blood, spleen in myeloid KLF4$^{-/-}$ mice, 24h post infection

The above-described *in vitro* results indicate that KLF4 affects inflammatory mediator liberation rather than affecting the pneumococcal killing ability of neutrophils. To investigate how these functional alterations influences the bacterial load *in vivo*, a mouse pneumonia model was used. Initially, colony forming unit (CFU) in the lungs, blood and spleen were analyzed. In the lungs (Fig. 14A), the KLF4$^{-/-}$ mice had significant higher bacterial load (around two log scales) 24h after infection with *S. pneumoniae* NCTC 7978 than the KLF4$^{+/+}$ mice. KLF4$^{-/-}$ mice also showed significantly higher bacterial load in the blood with respect to the KLF4$^{+/+}$ mice (Fig. 14B, around half log scale).The tendency was similar in the spleen

(Fig. 14C, around 3.5 log scales). The CFU results were further confirmed when the whole lung samples, after fixation, were stained with *S. pneumoniae* antibody and it was found that the amount of bacteria in the KLF4$^{-/-}$ mice was 2 times than that of KLF4$^{+/+}$ mice, 24h post infection with NCTC 7978 (Fig. 14D). Similarly, KLF4$^{-/-}$ mice showed significantly higher bacterial load in lungs than KLF4$^{+/+}$ mice, 48h post infection (Fig. 14E, around 4 log scales).

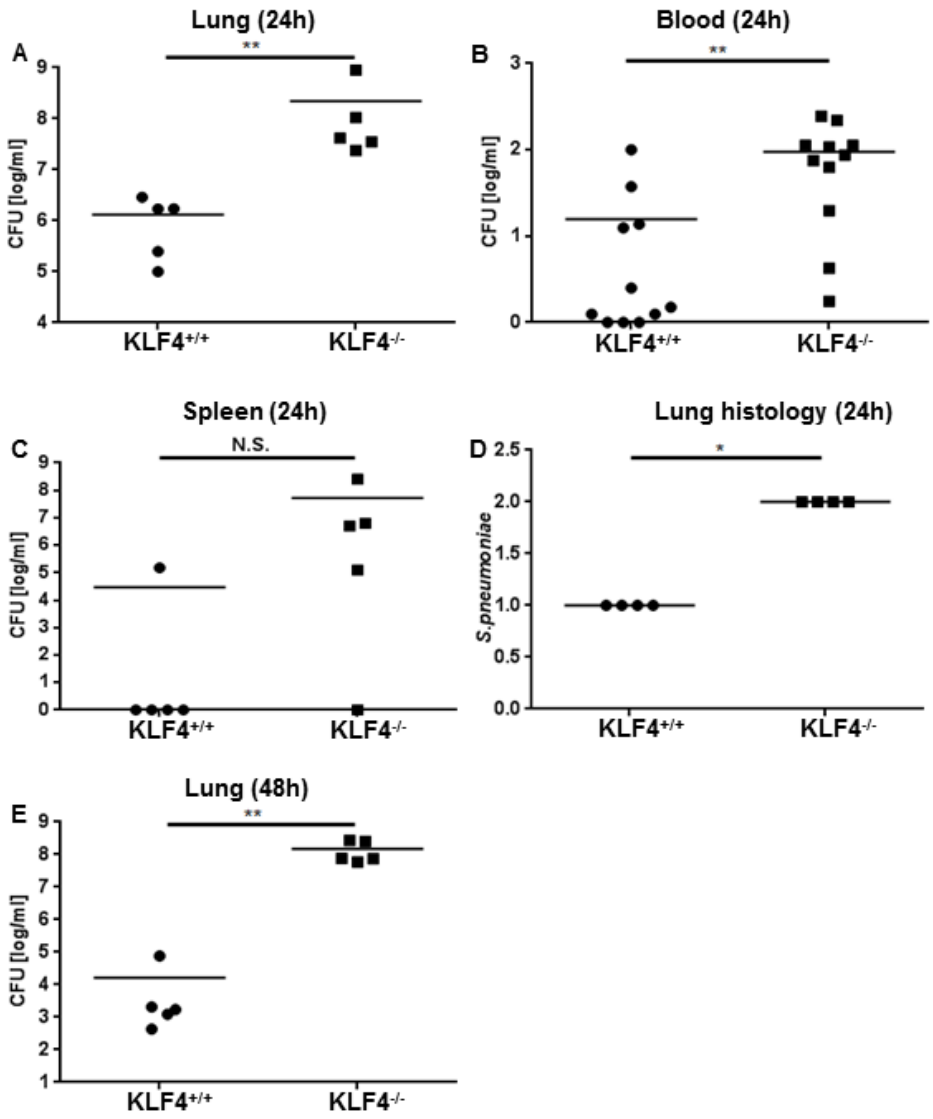


Fig. 14 KLF4$^{-/-}$ myeloid cells leads to higher bacterial load.

C57BL/6 mice were infected transnasal with 5x10^5 *S. pneumoniae* (Strain NCTC 7978) and were sacrificed 24h or 48h post infection. The lung, spleen samples (after homogenization in 1x PBS) and blood samples (collected in K_2EDTA tubes) were plated on blood agar plates in serial dilution. The blood agar plates were incubated overnight at 37°C, 5% CO_2. The CFUs were counted the next day. The results are from five mice in each group (Fig. 14A, C, and E) and eleven mice in each group (Fig. 14B). For the histopathology analysis, four mice were used in each group (Fig. 14D). Each lung section were stained with *S. pneumoniae* antibody and semi-quantitatively evaluated under light microscope for positive cells. The results were analyzed by Mann-Whitney

U test and statistically significant differences were characterized using graphpad prism software, by asterisks as follows: P< 0.05 *; P<0.01 **. N.S. = not significant.

6.2.2 Lower pro-inflammatory cytokine and higher anti-inflammatory cytokine in BALF of myeloid KLF4$^{-/-}$ mice, 24h post infection

Usually, the measured cytokine levels correspond to the bacterial load in the lung or in the blood: the more bacteria, the more cytokine response[55,56]. Surprisingly, although there were more bacteria in KLF4$^{-/-}$ mice compared to the KLF4$^{+/+}$ mice, there was significantly less TNF-α (Fig. 15A), IL-1β (Fig. 15B), KC (Fig. 15C) while significantly more anti-inflammatory cytokine such as IL-10 (Fig. 15D) in BALF of KLF4$^{-/-}$ mice in comparison to KLF4$^{+/+}$ mice, 24h after infection with *S. pneumoniae* NCTC 7978. In the PBS treated mice in both the KLF4$^{-/-}$ and KLF4$^{+/+}$ groups, no cytokines could be detected in the BALF.

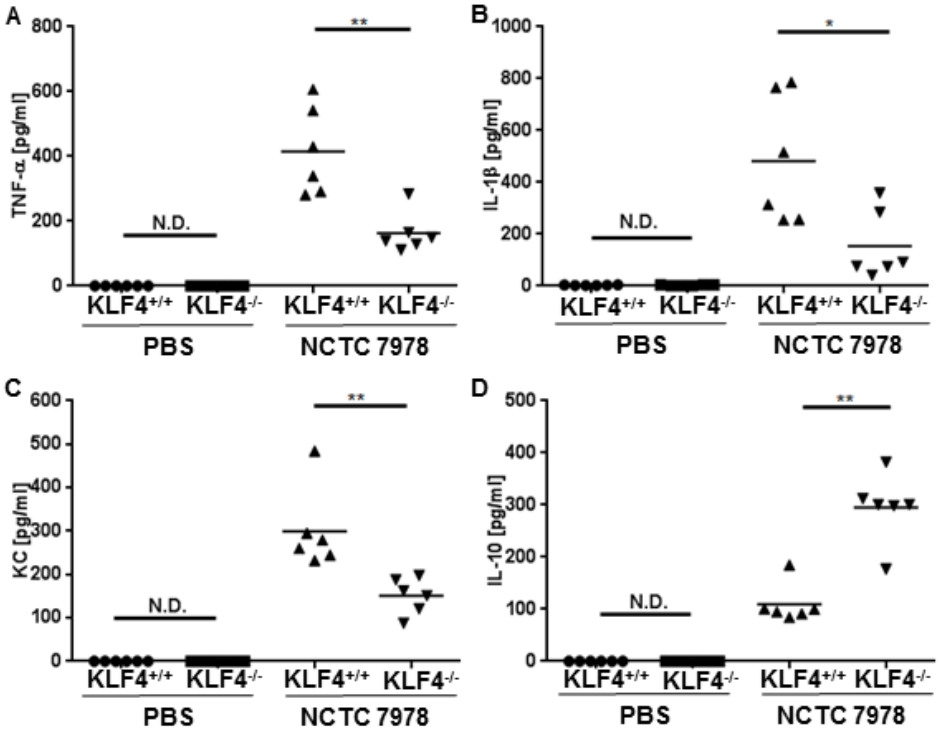


Fig. 15 Analysis of the cytokine levels in the BALF of PBS and S. pneumoniae infected mice.

Cytokine ELISA was done using commercially available kits to detect the cytokine levels in the BALF. C57BL/6 mice were infected transnasal either with 5x10^5 *S. pneumoniae* (Strain NCTC 7978) or with PBS and were sacrificed 24h post infection and BALF was taken after flushing the lung twice with PBS. The results are from six mice in each group and statistics were done by Mann-Whitney U test. P<0.05 * and P<0.01 ** was considered as the level of significance. The cytokine levels in the BALF of PBS infected mice in both the groups were below the detection limit (N.D. - Not Detectable).

6.2.3 Lower pro-inflammatory cytokine and higher anti-inflammatory cytokine levels in plasma of myeloid KLF4$^{-/-}$ mice, 24h post infection

Just like the BALF, KLF4$^{-/-}$ myeloid cells led to production of significantly less pro-inflammatory cyto-/chemokines (TNF-α, KC and IL-1β in Fig. 16A, B and C respectively) and significantly more anti-inflammatory cytokine in the plasma (IL-10 in Fig. 16D) although there were much more bacteria in the blood of KLF4$^{-/-}$ mice. No cytokines could be detected in the in the PBS treated KLF4$^{-/-}$ and KLF4$^{+/+}$ mice.

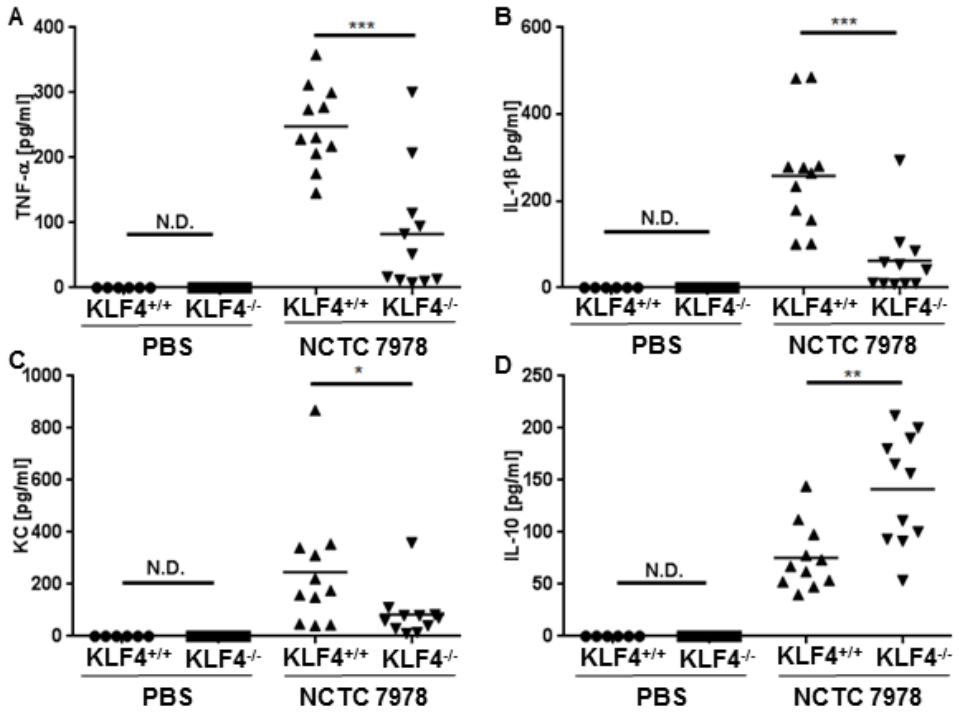


Fig. 16 Analysis of the cytokine levels in the plasma of PBS and S. pneumoniae infected mice.

Cytokine ELISA was done using commercially available kits to detect the cytokine levels in the blood. C57BL/6 mice were either infected transnasal with 5x10^5 *S. pneumoniae* (Strain NCTC 7978) or with PBS and were sacrificed 24h post infection and blood was collected in K_2EDTA tubes and these samples were then centrifuged for the collection of plasma which was used for further analysis . The results are from six mice in each group for the PBS mice (Fig. 16A-D) which were below detection limit (N.D. - Not detectable). For the NCTC 7978 infected category, eleven mice were used in each of KLF4$^{+/+}$ and KLF4$^{-/-}$ group (Fig. 16A-D). Graphs shown are analyzed by Mann-Whitney U test and $P<0.05$ *; $P<0.01$ ** and $P<0.001$ *** was considered as the level of significance.

6.2.4 Less neutrophil recruitment in the blood but not in the BALF of KLF4$^{-/-}$ mice, 24h post infection

Given that a lower pro-inflammatory cytokine profile was found in the KLF4$^{-/-}$ mice, it was expected to see less cell recruitment in BALF and plasma. Even though there was less

neutrophil recruitment in the blood of KLF4$^{-/-}$ mice in comparison to KLF4$^{+/+}$ mice (Fig. 17A), there were no differences in neutrophil recruitment between the KLF4$^{-/-}$ and KLF4$^{+/+}$ mice in the BALF (Fig. 17B), 24h after infection with *S. pneumoniae* NCTC 7978.

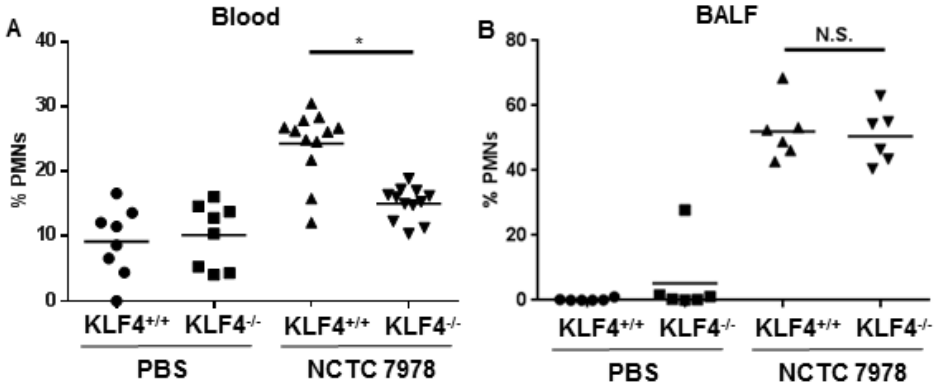


Fig. 17 Analysis of cell recruitment in the blood and BALF of PBS and S. pneumoniae infected mice.

C57BL/6 mice were either infected transnasal with 5x10^5 *S. pneumoniae* (Strain NCTC 7978) or with PBS and were sacrificed 24h post infection and BALF was taken after flushing the lung twice with PBS. Blood was collected in K_2EDTA tubes and analyzed using Scil vet abc hematology analyzer (Fig. 17A). BALF samples were analyzed using Fluorescence-activated cell sorting (FACS) (Fig. 17B). The results are from at least six mice in each group and were analyzed with one-way ANOVA followed by Dunn's multiple comparison test. $P<0.05$ * was considered as the level of significance. N.S. = not significant.

6.2.5 Myeloid KLF4$^{-/-}$ led to increased vascular permeability within the lungs during pneumococcal pneumonia

The presence of the bacteria, their released virulence factors (such as Ply) as well the as the resulting host innate immune response itself contributes to lung barrier failure with deleterious breakdown of the lung gas exchange in severe pneumonia[45,176]. To assess the pulmonary vascular permeability, mouse albumin (MA) ELISA was done with the respective BALF and plasma of each mouse to follow the leakage of the albumin molecule as a marker of barrier failure from the circulation into the airspace. It was observed that KLF4$^{-/-}$ mice have significantly higher vascular permeability (Fig. 18) with respect to KLF4$^{+/+}$ mice, 24h post infection with *S. pneumoniae* NCTC 7978. In the PBS treated mice, no increase in vascular permeability was detected in both groups, 24h post infection.

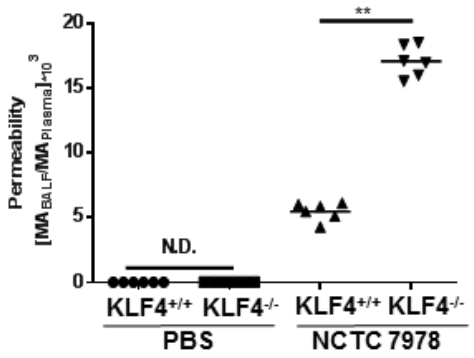
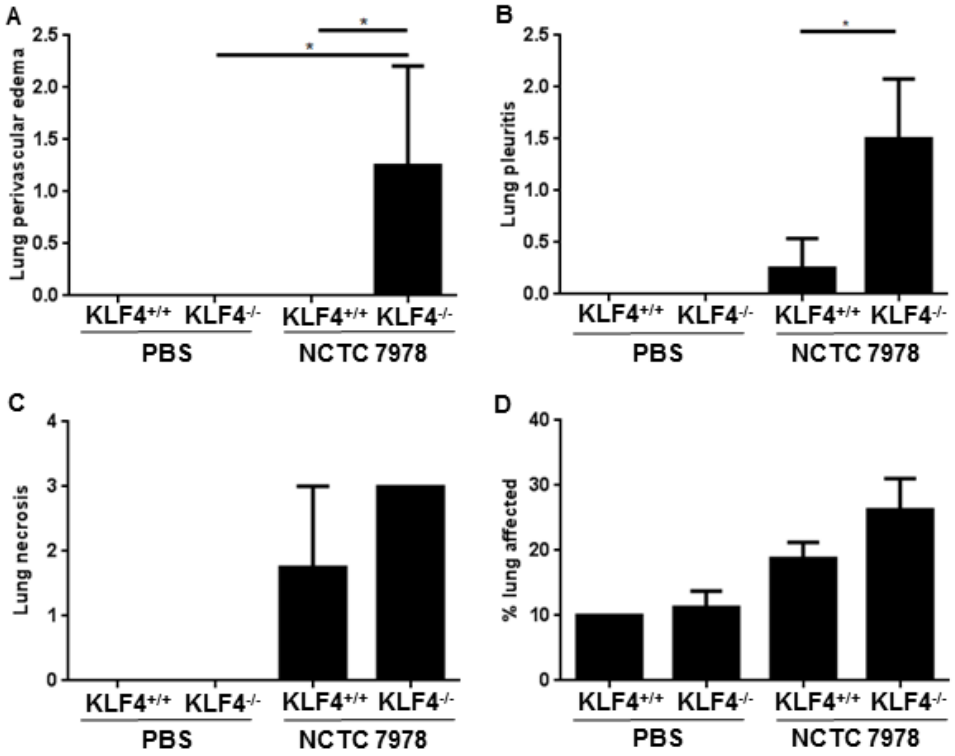


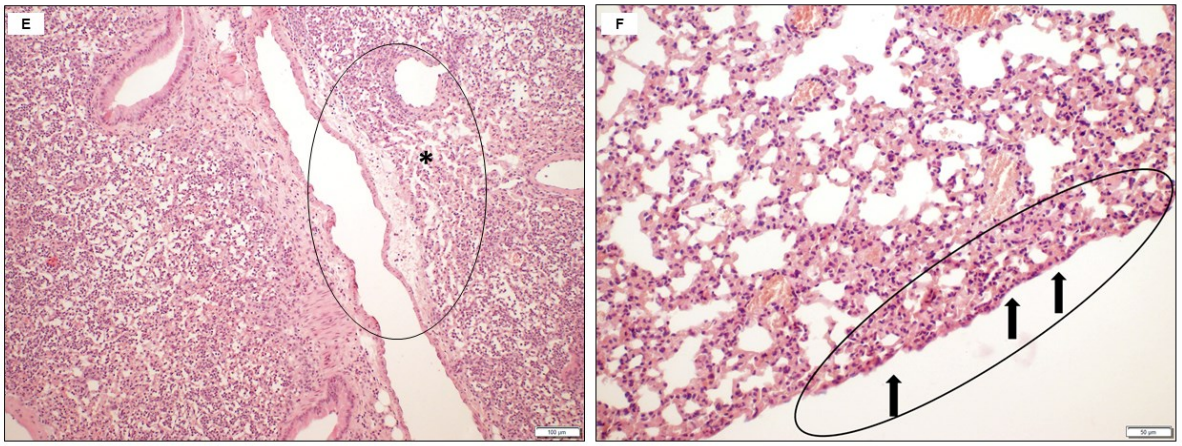
Fig. 18 Myeloid KLF4$^{-/-}$ led to increased vascular permeability during bacterial pneumonia.

C57BL/6 mice with myeloid KLF4$^{-/-}$ and KLF4$^{+/+}$ lineage were either infected transnasal with 5x10^5 *S. pneumoniae* (Strain NCTC 7978) or with PBS and were sacrificed 24h post infection. The quantification of vascular permeability was done by the ratio of respective BALF/Plasma ratio and multiplying it with 10^3. The results are shown from six mice in each group and were analyzed by Mann-Whitney U test and statistically significant differences were characterized using graphpad prism software, by asterisks as follows: $p<0.01$ **.

6.2.6 KLF4$^{-/-}$ mice have more perivascular edema, pleuritis, necrosis and more affected lung area, 24h post infection

KLF4$^{-/-}$ mice showed significantly more perivascular edema (Fig. 19A) and pleuritis (Fig. 19B) in the whole lung, 24h post infection with *S. pneumoniae* NCTC 7978. Myeloid KLF4$^{-/-}$ mice also showed a higher tendency of necrosis (Fig. 19C) and affected lung area (Fig. 19D) 24h after infection. Whole lung images were obtained to assess these lung inflammation scores. Representative pictures shown for lung perivascular edema (Fig. 19E), pleuritis (Fig. 19F) and necrosis (Fig. 19I) which were found vividly in KLF4$^{-/-}$ mice 24h post infection with *S. pnemoniae* but absent or comparatively found lesser in KLF4$^{+/+}$ mice (Fig. 19G and H). Representative pictures are also shown for whole lung images for both the KLF4$^{-/-}$ and KLF4$^{+/+}$ mice, PBS and NCTC 7978 infected (Fig. 19J-M).


A
Lung perivascular edema
2.5
2.0
1.5
1.0
0.5
0.0
*
*
KLF4+/+ KLF4-/- KLF4+/+ KLF4-/-
PBS
NCTC 7978
B
Lung pleuritis
2.5
2.0
1.5
1.0
0.5
0.0
*
KLF4+/+ KLF4-/- KLF4+/+ KLF4-/-
PBS
NCTC 7978
C
Lung necrosis
4
3
2
1
0
KLF4+/+ KLF4-/- KLF4+/+ KLF4-/-
PBS
NCTC 7978
D
% lung affected
40
30
20
10
0
KLF4+/+ KLF4-/- KLF4+/+ KLF4-/-
PBS
NCTC 7978


E
*
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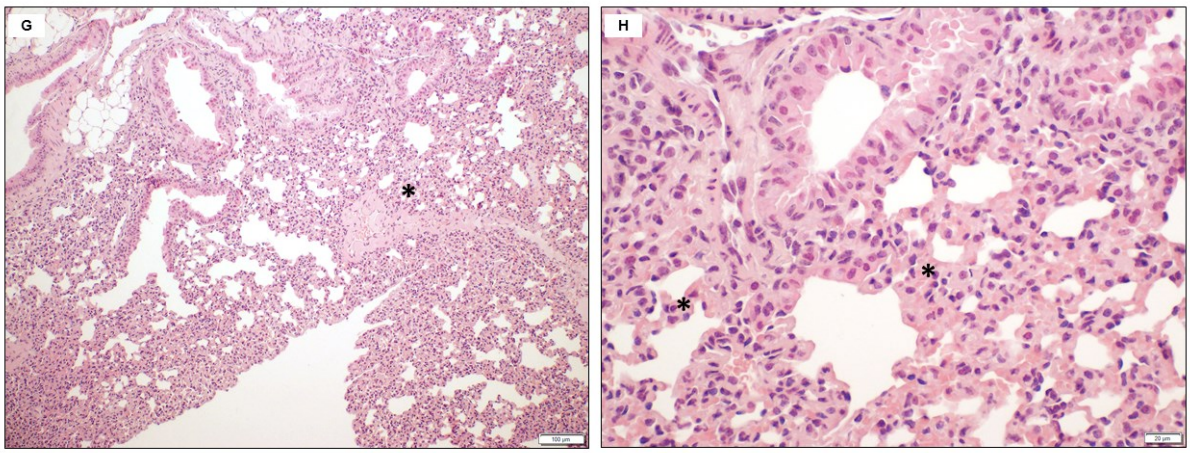
G
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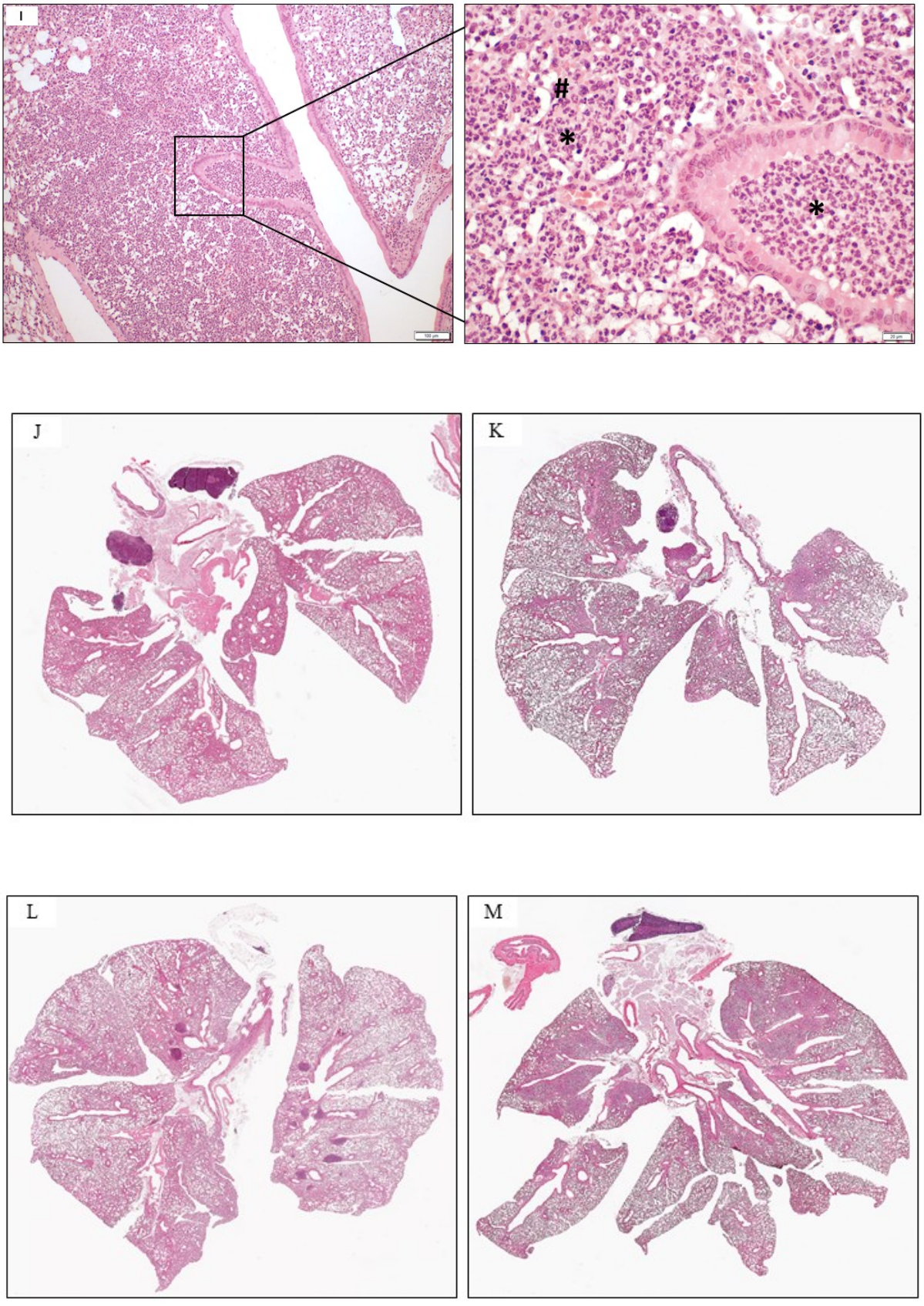


Fig. 19 Analysis of lung inflammation score.

C57BL/6 mice were infected transnasal either with $5x10^5$ *S. pneumoniae* (Strain NCTC 7978) or with PBS and were sacrificed 24h after the infection and whole lung samples were fixed in 4% PFA for up to 48h. The samples

were then stained with Hematoxylin/Eosin for further analysis. The results are shown as Mean ± SD in Fig. 19A-D (above) are from four mice in each group and were analyzed with one-way ANOVA followed by Dunn's multiple comparison test. Statistical significance was considered as follows: P<0.05 *. (middle) Representative pictures of various lung inflammation scores found in KLF4$^{-/-}$ mice (Fig. 19E, F and I) but absent or found to a very less extent in KLF4$^{+/+}$ mice (Fig. 19G, H). In Fig. 19E, KLF4$^{-/-}$ mice infected with NCTC 7978 shows perivascular edema (*) at 10x magnification. In Fig. 19F, similarly, KLF4$^{-/-}$ mice shows pleuritis (↑) at 40x magnification. In Fig. 19G and H, KLF4$^{+/+}$ mice infected with NCTC 7978 shows thickening of alveolar membranes (*) at 10x and 40x magnification respectively, indicating interstitial pneumonia. In Fig. 19I, KLF4$^{-/-}$ mice, 24h after infection with NCTC 7978 shows necrosis of alveolar membrane (#) with neutrophil infiltration (*), indicating suppurative bronchopneumonia (*). (below) Whole lung images of PBS treated and NCTC 7978 infected KLF4$^{-/-}$ and KLF4$^{+/+}$ mice were obtained as given in representative images for KLF4$^{+/+}$ PBS (Fig. 19J) and NCTC 7978 infected mice (Fig. 19K) and also KLF4$^{-/-}$ PBS (Fig. 19L) and NCTC 7978 mice (Fig. 19M) to assess the histopathology associated with the inflammation during pneumococcal pneumonia.

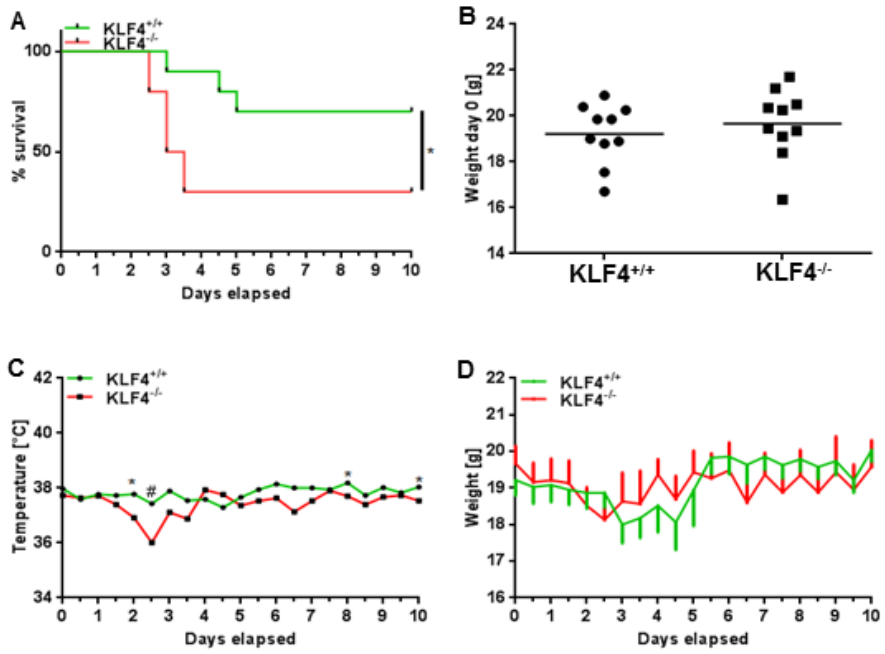
6.2.7 No impact of myeloid KLF4$^{-/-}$ on other visceral organs, 24h post infection

We hypothesized that higher bacterial load in the blood of the myeloid KLF4$^{-/-}$ might affect other organs in the body. As a result, other organs such as brain, thymus, heart, spleen, liver, small intestine, large intestine, kidneys of KLF4$^{-/-}$ and KLF4$^{+/+}$ mice have also been used for histopathological analysis. Intact organ samples from PBS and NCTC 7978 infected from both the KLF4$^{-/-}$ and KLF4$^{+/+}$ mice (n = four in each group of PBS and NCTC 7978), after fixation in 4% PFA were stained with hematoxylin and eosin and analyzed for inflammatory symptoms. However, no major differences or impact were found between the two groups, 24h after infection (data not shown).

6.2.8 Myeloid KLF4$^{-/-}$ mice show more clinical symptoms of sickness and had to be euthanized earlier compared to KLF4$^{+/+}$ mice

In the next step, the overall effect of myeloid KLF4 on pneumococcal pneumonia in mice was assessed. Experiments were done to investigate the impact of KLF4$^{-/-}$ on the survival of mice after infection with $5x10^4$ CFU/ml. The lower dosage was used as the mice were monitored 10 days post infection to see whether the KLF4 KO had any impact on the overall survival. Doehn et al have previously used the same experimental setup where the C57BL/6 mice had been treated with Cpl-1, 24h after infection with *S. pneumoniae* and was monitored over a period of 10 days (240h) to assess their survivability[177]. In Fig. 20A, it is seen KLF4$^{-/-}$ mice had to be euthanized earlier and more with respect to KLF4$^{+/+}$. They generally reached the human endpoints within 2.5 to 3.5 days after infection while KLF4$^{+/+}$ mice between 3 to 5 days after infection. The weight of both KLF4$^{-/-}$ and KLF4$^{+/+}$ mice at the beginning of experiment were same (Fig. 20B). There were major differences in temperature between

KLF4$^{-/-}$ and KLF4$^{+/+}$ mice (Fig. 20C) but not any differences in their weight over the period of 10 days (Fig. 20D). The fall in body temperature in KLF4$^{-/-}$ mice was especially between 2 - 2.5 days after the infection. Various clinical symptoms, which showed that KLF4$^{-/-}$ mice are sicker than the KLF4$^{+/+}$ mice, are shown in Fig. 20E-J. These clinical symptoms are from experiments where the animals have been infected with a higher dosage of NCTC 7978 ($5x10^5$ CFU/ml). In these experiments, the animals had to be monitored up to 48h (end of the experiments). Certain clinical symptoms such as accelerated breathing rate (Fig. 20E and F) were shown by KLF4$^{-/-}$ mice much earlier (36h time point) with respect to KLF4$^{+/+}$ mice (seen only at 48h time point). This was also observed with the mice reacting to external stimuli. KLF4$^{-/-}$ mice started to be less responsive to external stimuli at 36h time point (Fig. 20G) while the same was observed with KLF4$^{+/+}$ only at 48h time point (Fig. 20H). In addition, KLF4$^{-/-}$ mice always showed much more raised fur be it at 24h or 48h (Fig. 20I and J respectively) with respect to KLF4$^{+/+}$. All the parameters and guideline for these clinical symptoms including body weight and temperature are well defined in the LAGeSo proofed monitoring sheet. Animals were always sacrificed when their level of pain reached the human end points in consultation with department-authorized veterinarian.



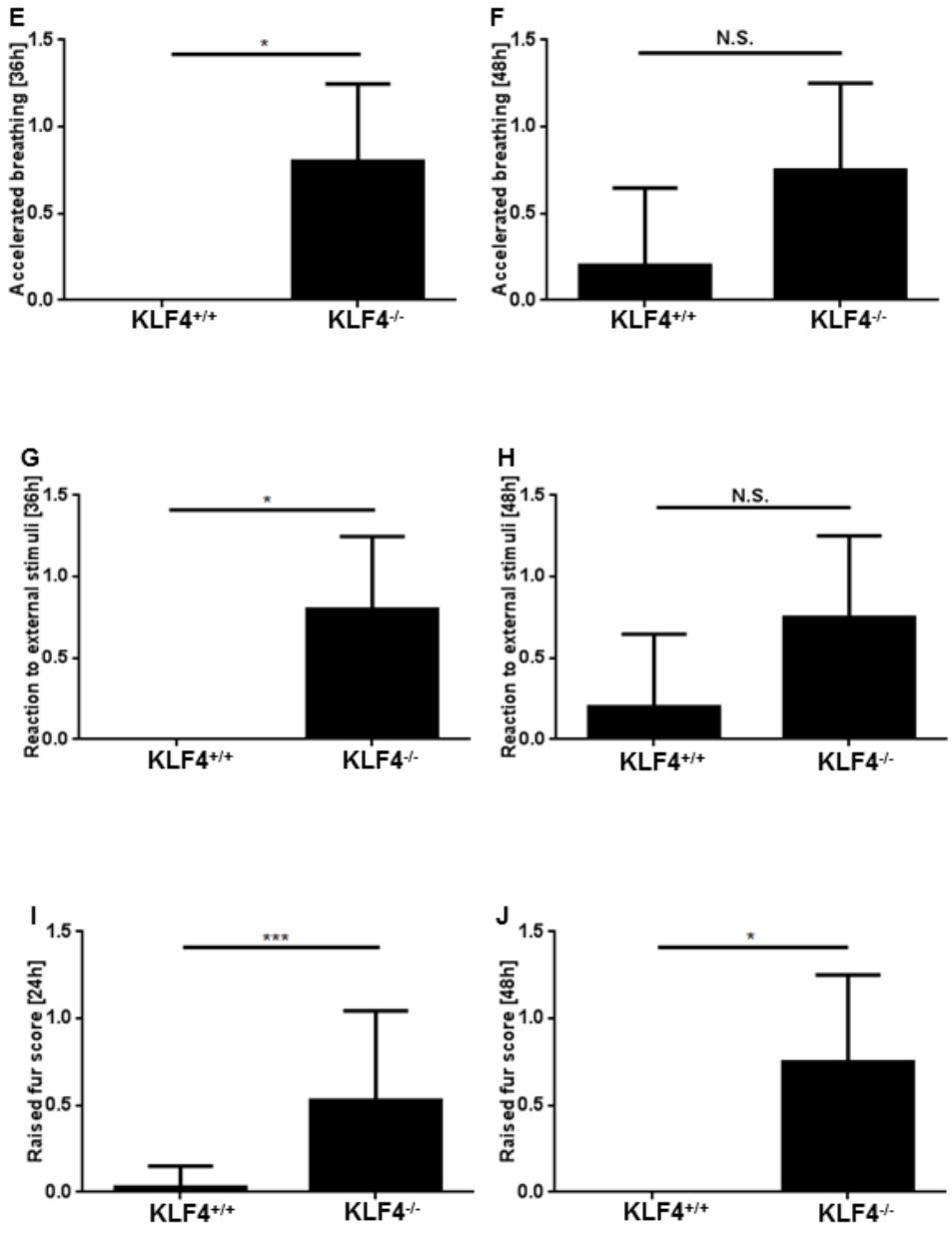


Fig. 20 Myeloid KLF4$^{-/-}$ mice reach earlier the human end points post infection and shows early and higher clinical symptoms of sickness.

Fig. 20A shows that KLF4$^{-/-}$ mice had to be euthanized earlier compared to KLF4$^{+/+}$ mice (each group n = ten). The weight, temperature and clinical symptoms (such as raised fur, response to external stimuli, movement etc.) were monitored every 12h after infection. The mice were euthanized when their level of pain reached the human end point in consultation with a department-authorized veterinarian. The result shows the percent of animal survival every 12h in each group over the period of 10 days (240h). The graph is analyzed using Log-rank (Mantel Cox) test. Statistical significance is defined as $P<0.05$ *. Fig. 20B shows the weight of individual mice in each group along with the mean weight of KLF4$^{-/-}$ and KLF4$^{+/+}$ at day zero. Fig. 20C, D shows variation in body temperature (C) and weight (D) in both groups over the period of 10 days. In Fig. 20C, multiple t-test has

been done with the help of graphpad prism software and the statistical significance are given as follows: P<0.05 * and p = 0.063 #.

In Fig. 20E-J the clinical symptoms from animals infected with higher dosage were also assessed. $KLF4^{-/-}$ mice showed earlier symptoms of accelerated breathing rate at 36h (Fig. 20E, n = five in each group) than $KLF4^{+/+}$ mice which showed only at 48h (Fig. 20F, n = four to five in each group). $KLF4^{-/-}$ mice started to show lower responses to external stimuli at 36h (Fig. 20G, n = five in each group) than $KLF4^{+/+}$ mice which showed at 48h (Fig. 20H, n = four to five in each group). Higher scores of $KLF4^{-/-}$ in the graph of reaction to external stimuli imply that they are more 'not' responsive to external stimuli. $KLF4^{-/-}$ mice showed much more raised fur not only at 24h (Fig. 20I, n = seventeen in each group) but also at 48h (Fig. 20J, n = four to five in each group). All the graph are shown as Mean ± SD and analyzed using Mann-Whitney U test and P<0.05 * P<0.001 *** was considered as the level of significance. N.S. = not significant.

7 DISCUSSION

Bacterial infections are the most prevalent disease in the human lower respiratory tract[5]. With the advent of MDR bacterial strains, treatment of the lower respiratory tract infections has become a big challenge[91]. Bacterial pneumonia is one of leading causes of death worldwide and it is most frequently caused by *S. pneumoniae*[7,8,9]. Neutrophils are not only important component of the innate immune system but also plays a key role in the regulation of adaptive immunity[85,178,179]. Neutrophils spearhead the host responses to pneumococcal pneumonia which results in an acute inflammation in the lung[91,180]. The important role of neutrophils during infections can be underscored when patients with neutrophil deficiencies such as neutropenia suffer from severe complications, which even lead to death[181]. Previous reports have also suggested that how neutrophil depletion can cause reduced clearance of *S. pneumoniae*[91,92]. Thus, if the neutrophils were functionally efficient, then the host would benefit from better bacterial clearance and in turn recovery. However, the transcriptional regulation for the proper functioning of neutrophils during pneumonia caused by *S. pneumoniae* remains poorly understood.

7.1 *In vitro*

In Fig. 9 and 10, it was demonstrated that both human and mouse neutrophils express KLF4 when exposed to pneumococci indicating that KLF4 in neutrophils during *S. pneumoniae* may be of functional relevance. Since previous reports have suggested that KLF4 can be induced (by infection) in some other cells such as human lung epithelial cells[136,167], human monocyte derived DCs[182], BMMs[163] and peritoneal mouse macrophages[163,183], KLF4 may act as an important regulatory transcription factor in bacterial infections. Though the present study mainly involves a murine model, human blood neutrophils have also been used in the *in vitro* study to a) address the induction in genetic diverse mammals and b) humans. The induction of KLF4 was much more with the unencapsulated variant (R6x). In the previous *in vitro* studies, there was an induction of KLF4 in BMMs after stimulation with *S. pneumoniae*. Viable, replicating bacteria and released DNA are the responsible factors for the KLF4 induction in BMMs[169]. The thick polysaccharide capsule cover up the important components of the cell wall and this protects the bacteria against phagocytosis[15]. However, myeloid KLF4 deficiency did not alter phagocytosis of pneumococci (see below) in the experiments performed in this study. The capsule however prevents bacteria from being entangled in NETs[15]. KLF4 is spontaneously upregulated with Dachshund homolog 1 (DACH1) which in turn regulates cyclin dependent kinase (cdk) 4/6, a key machinery required for NETs formation[184,185]. Since a lower capsule content increases the chance of the bacteria being entangled in NETs which

may be influenced by KLF4 via the DACH1-cdk4/6 axis, this may be a postulated for the higher induction of KLF4 with the unencapsulated pneumococci. In addition, since unencapsulated pneumococci are responsible for more expression of KLF4 than encapsulated pneumococci, it can also state that the inducing factor for KLF4 is a pneumococcal cell wall component which is partly hidden by the capsule.

Of note, the presented Western blots in Fig. 9 and 10 suggested that there was no KLF4 induction at basal level which also has been reported at mRNA levels from other groups[186]. In contrary some groups have suggested that mouse bone marrow monocytic cells and peripheral blood monocytes express KLF4 at basal conditions[162,183]. In contrast to the increased induction of KLF4 with the unencapsulated pneumococci (as stated above) there was nearly no KLF4 induction when the cells were stimulated with pneumococci lacking the virulence factor autolysin (LytA) as given in Fig. 10. Further, unlike in epithelial cells, there is no induction of KLF4 with bacterial DNA alone[136] but the signal was partly back when the cells were stimulated with the combination of pneumococcal strain R6xΔlytA and bacterial DNA, the inducing factor for KLF4 in macrophages[169]. It has been reported that *S. pneumoniae* autolysin LytA (N-acetylmuramoyl-L-alanine amidase) activates immunomodulatory signaling pathways in macrophages by releasing bacterial cell wall constituents such as peptidoglycan and diconic acid[187]. Moreover, autolysis is important for the release of bacterial DNA[188]. The released DNA from pneumococci has also been stated to induce interferon-β (IFN-β) during infection[189]. Therefore, the expression of KLF4 in neutrophils is dependent on autolysis and may be partly on the release of bacterial DNA during the infection.

Previous literature has suggested the recognition of *S. pneumoniae* is mediated among other receptors via Toll-like receptors (TLR2, probably TLR4 and TLR9)[15,21,190,191,192]. However, in the present study when neutrophils were stimulated with the TLR2 ligand MALP2 (as TLR2 agonist) as given in Fig. 9B, there was no induction of KLF4 noted in human neutrophils. The pneumococcal cell wall components such as lipoteichoic acid and or lipoproteins are recognized by TLR2, which does not seem to have a role in KLF4 induction in neutrophils. Comparable observations have been reported by Taylor and colleagues, who demonstrated that the stimulation of embryonic stem cells with Pam3Cys, another TLR2 ligand, was not able to induce KLF4 expression[193].

In Fig. 9B, we also see that when neutrophils were stimulated with LPS as TLR4 agonist, there was no expression of KLF4. These results are in line with the findings of Liao et al. He and his coworkers had reported that LPS treated macrophages showed a reduction in KLF4

expression[163]. In contrast Liu et al. had pointed out the induction of KLF4 in macrophages after treatment with LPS[194]. The difference in finding may be because of the experimental setup: Liao et al. had used peritoneal macrophages and bone marrow derived macrophages from mice, Liu et al. had used macrophage cell line while the present study was done using human primary neutrophils. Additionally, Ply an important virulence factor of *S. pneumoniae*, released after autolysis may initiate inflammatory response through TLR4[15] but TLR4 seems not have a role in expression of KLF4 in human neutrophils in the present study.

Human neutrophils were also stimulated with CpG as TLR9 agonist but there was no expression of KLF4 noted (see Fig. 9B). In contrast to epithelial cells, as given in Fig. 10 we have also seen that *S. pneumoniae* DNA as a TLR9 ligand was not able to induce KLF4 (above) in mouse bone marrow neutrophils and human neutrophils. Thus, the partial induction of KLF4 by the bacterial DNA is rather independent of TLR9 but may be through an unknown receptor as it has previously been reported about the recognition of DNA by RAW264.7 macrophages via TLR9 and an unknown DNA sensor in the cell[192]. These data support a highly differentiated cell type–specific regulation of KLF4 expression and an underlying hitherto only partly know signaling pathway.

The use of recombination systems such as Cre/loxP is a powerful tool for induction or suppression of genes in a cell/tissue specific manner in different mice models. With the conditional induction or suppression of the gene of interest, it helps us to find the cell specific role of that gene. The LyzMcre system was effective in ~90% knockdown in blood neutrophils (mature cells) but did not show any effect on immature neutrophils from the bone marrow or other WBCs as given in Fig. 11. The findings support the working principle of the LyzMcre system which states that it is effective only in mature myeloid cells and does not affect lymphocytes[170].

In the *in vitro* phagocytosis assay in Fig. 12, no differences in bacterial killing were observed between the $KLF4^{-/-}$ and $KLF4^{+/+}$ mice. However, Liao et al. have previously reported that KLF4-deficient macrophages have enhanced bactericidal activity[163]. The difference in the outcome of the present findings may be because they used different bacteria (*S. pneumoniae versus Staphylococcus aureus*)[163]. In line with the observation in that study, Shen et al. have reported that bone marrow derived KLF4 KO neutrophils failed to kill *E. coli*[166]. *S. pneumoniae* initially interacts with the alveolar epithelial cells and the resident macrophages within the lung which in turn induces the chemotactic gradient for neutrophil sequestration at the site of infection[91]. Additionally report suggest that *S. pneumoniae* can reduce its capsule

content once it encounters airway epithelial cells[20]. Previous report have suggested that phagocytosis of *S. pneumoniae* by neutrophils is dependent on its capsule[195]. In all probable, the neutrophils, after sequestration to the site of inflammation, may interact with *S. pneumoniae* with low capsule content. Since we have already seen an increased KLF4 expression with unencapsulated *S. pneumoniae* R6x in comparison to the wild type encapsulated *S. pneumoniae* strain D39 (see above), as a result, two different strains of *S. pneumoniae* have been used for the phagocytosis assay to assess the role of myeloid KLF4 during phagocytosis with respect to bacterial capsule content.

Unlike the bacterial killing, the KLF4$^{-/-}$ myeloid cells have a change in the cytokine profile *in vitro* as given in Fig. 13. In this study, it could be shown that KLF4$^{-/-}$ neutrophils have less pro-inflammatory cytokines such as TNF-α and KC, 16h after stimulation with *S. pneumoniae* wildtype strain D39. The D39 strain have been used for the *in vitro* cytokine analysis because it has previously been reported that the capsulated variant of *S. pneumoniae* (D39) triggered much more cytokine response such as TNF-α, IL-6 and KC in comparison to the unencapsulated strain (D39Δcps). As a result, the capsule is needed for the early induction of cytokines[196]. Though the average life span of neutrophils in circulation is 8-12h but their life span greatly increases in the presence of pro-inflammatory mediators[197]. Additionally, Sangaletti et al. have co-cultured neutrophils isolated from mice along with myeloid DCs for 16h[198]. The changes in pro-inflammatory cyto-/chemokine profile are in line with reports from Liu et al: they have reported that RAW264.7 macrophages express KLF4 and this expression of KLF4 regulates pro-inflammatory cytokines such as IL-1β[194]. Also, Feinberg and Kaushik et al. had previously hypothesized about the pro-inflammatory phenotype in myeloid cells such as macrophages and also in monocytic microglial cells[165,199]. Other studies have also provided insights into how KLF4 can fine-tune the inflammatory cytokines in different cell types. Tetreault et al. had observed KLF4 induces pro-inflammatory cytokines such as TNF-α and IL-1α via modulation of NF-κB in keratinocytes[200]. Another report had demonstrated that KLF4 is responsible of pro-inflammatory cytokines such as IL-6 in fibroblast-like synoviocytes of rheumatoid arthritis patients[201]. On the other hand, other groups have studied the anti-inflammatory role of KLF4. Hamik et al. for example, have shown that KLF4 confers anti-inflammatory phenotype in human umbilical vein endothelial cells by reducing pro-inflammatory cytokine production such as IL-6 and C-reactive protein[202]. Another study had reported that KLF4 nullifies the induction of macrophage migration inhibitory factor (MIF) by transforming growth factor-β1 in renal tubular cells[203]. It is interesting to mention that MIF has been reported to be expressed in different cell types such as macrophages, PMNs and

epithelial cells[204,205,206]. This MIF is responsible for induction of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1 from macrophages[207]. These data support a cell type–specific function of KLF4. In two different publications, Zahlten at al. have pointed out how KLF4 can regulate the NF-κB pathway and in turn control the cytokine profile during *S. pneumoniae* induced pneumonia[136,167] in human broncho-epithelial cells. While KLF4 is pro-inflammatory in myeloid cells as have been found in the present study and also has been reported earlier, whereas in the lung epithelial cells, loss of KLF4 during pneumococcal pneumonia enhanced pro-inflammatory cytokine response and reduced anti-inflammatory IL-10 secretion[136,167]. Taken together, the results point out towards a pro-inflammatory role of KLF4 in myeloid cells but an anti-inflammatory role in at least broncho-epithelial cells during pneumococcal pneumonia. Thus, it can be speculated that during bacterial pneumonia, KLF4 in myeloid cells and KLF4 in epithelial cells eliminates the pathogen and controls the inflammation respectively for maintenance of homeostasis. The *in vitro* findings are depicted in Fig. 21.

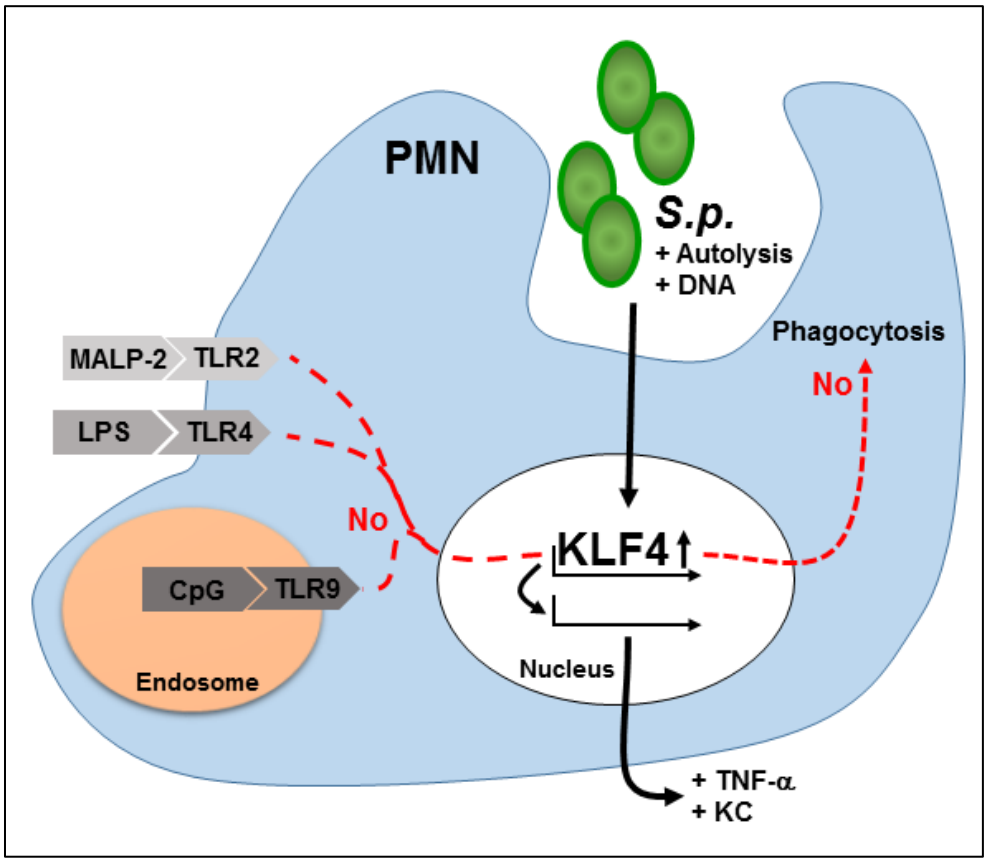


Fig. 21 Pneumococci-dependent expression and function of KLF4 in neutrophils.

The stimulation of neutrophils with MALP2 (TLR2 agonist), LPS (TLR4 agonist) and CpG (TLR9 agonist) did not induce KLF4 expression in neutrophils. However, bacterial (*S.p.*= *Streptococcus pneumoniae*) autolysis and bacterial DNA are at least in part responsible for KLF4 expression in neutrophils and this leads to production of

pro-inflammatory cytokines such as TNF-α and KC. It is also noteworthy that KLF4 has no impact on the phagocytic capability of the neutrophils on bacteria.

7.2 *In vivo*

After the identification of KLF4 over more than twenty years back[208], further experiments had been done to assess the physiological role of this transcription factor. Genome wide KLF4 KO in mice has a lethal phenotype because of the lack of establishment of skin barrier[156]. As a result, there was a need for cell specific or organ specific KLF4 KO in mice to understand the tissue specific role of KLF4. Various models of KLF4 KO in a cell specific or organ specific manner have been used: for example, An et al. had demonstrated KLF4 KO in T cells to study T-cell development[209]. Yu et al. had used KLF4 KO lungs in murine model to study its role during lung cancer[210]. However, cell specific or organ specific KLF4 KO mice have rarely been used in bacterial infection model based on extensive literature review. One such model is used by Tussiwand et al. where they infected mice lacking KLF4 in cDCs with *Citrobacter rodentium*[211]. A recent study has also demonstrated the role of myeloid KLF4 KO during *E. coli* (a gram negative bacterium) infection[166]. In this *E. coli* induced sepsis mice model, it was shown that myeloid KLF4 has a pro-inflammatory phenotype. Myeloid KLF4 KO mice had higher bacterial burden with respect to WT mice, which was due to the lower pro-inflammatory cytokine production by the myeloid cells after infection with *E.coli*. Consequently, there was less neutrophil recruitment in the KLF4 KO mice. Due to the higher bacterial load, there was higher mortality in KLF4 KO mice in comparison to the WT mice.

The choice of animal model and the bacterial strain to be used for the *in vivo* study is an important consideration for experiments with pneumococcal pneumonia. Rat and rabbit models are uncommonly used in comparison to mice models. Among the mice models, various inbred strains such as BALB/c, C57BL/6 and CBA/J model have been used for studies in *S. pneumoniae* induced pneumonia. In principle, there are two ways in how the lungs in the mice can be infected-intratracheal or intra-/transnasal. The mice model used in the present study have always been infected transnasal, as the mode of infection intratracheal is complex and invasive technique. Additionally, another advantage of the transnasal application of pneumococci is that it mimics the natural way the pathogen enters the human system[212]. Another important decision is the choice of bacterial strain to be used for the *in vivo* study. The different strains have been used for *in vitro* and *in vivo* studies is based on literature review. The findings by Orihuela et al. suggested that in *in vivo* infection model, D39 (serotype 2) was responsible for high-grade sepsis while A66 (NCTC 7978, serotype 3) was responsible for development of pneumonia in mice[213]. The capsule is one of the most

important virulence factor of *S. pneumoniae* and the higher capsular content determines the immune responses during pneumococcal pneumonia. Serotype 3 was chosen for the *in vivo* model instead of the serotype 2 because the serotype 3 has much more thick capsule while the serotype 2 is lowly encapsulated[20,214]. Not only does the *S. pneumoniae* serotype contribute to the outcome of the disease but also the host genetic factors contribute to the outcome of bacterial pneumonia. This is evident, based on different outcomes when different mice models are infected with the same *S. pneumoniae* strain[215]. Of the different types of mice model, serotype 3 strain has been commonly used for the C57BL/6 mice[212]. Taken together, the choice of animal model and the bacterial strain are important factors for studying pathogenesis of pneumonia.

We could see in our model, a steep change in bacterial load in mice, deficient in KLF4 in the myeloid cells. KLF4$^{-/-}$ mice had higher bacterial load in the lungs, blood and in the spleen at 24h post infection compared to the KLF4$^{+/+}$ mice from the CFU experiments in Fig. 14, which is supported by a recent report[166]. In this publication, Shen et al. had reported higher bacterial load in the blood and peritoneal fluid of myeloid KLF4 KO mice with respect to WT mice in an *E. coli* sepsis model. The CFU result could be further ascertained when the whole lung samples for histology analysis were stained with *S. pneumoniae* antibody as given in Fig.14D. The results of this histology experiment revealed that KLF4$^{-/-}$ mice had 2 times the bacteria than KLF4$^{+/+}$ mice. Overall, loss of myeloid KLF4 seem to impair the local limitation of bacterial growth and increase the risk of bacterial outgrowth.

Infection of pneumococci in the lung in a murine model is associated with increased cytokine level in the BALF and recruitment of neutrophils with a concurrent growth of bacteria in the alveolar space between 4h to 24h post infection[55]. As a result, higher the bacterial load, higher cytokine level is expected. In spite of the higher bacterial load, KLF4$^{-/-}$ mice in the herein discussed experiments had lower pro-inflammatory cytokines and higher anti-inflammatory cytokine. KLF4$^{-/-}$ mice showed lower levels of pro-inflammatory cytokines such as TNF-α, IL-1β and chemokine such as KC while had increased anti-inflammatory cytokine such as IL-10. The lower pro-inflammatory cytokines and enhanced anti-inflammatory cytokine was seen not only in the BALF but also in the plasma, 24h post infection as seen in Fig. 15 and 16 respectively. The results support the hypothesis and by findings from Shen et al. which shows that pro-inflammatory factors such as myeloidperoxidase (MPO) and matrix metallopeptidase (MMP)-9 are produced to a less extent in the myeloid KLF4 KO mice during infection[166]. In addition, *in vitro* studies supported a potential pro-inflammatory phenotype of KLF4 positive cells in BMMs: KLF4 KO in BMMs decreased TNF-α, IL-1β, IL-6 while increased IL-10

production during pneumococcal pneumonia[169]. The low TNF-α in the BALF and plasma of the KLF4$^{-/-}$ mice *in vivo* may be because of the dual impact of KLF4 in the neutrophils and the BMMs (as seen from the *in vitro* experiments). Likewise, the less IL-1β and more IL-10 production may be because of their regulations by KLF4 in BMMs. Additionally, the low KC production in the KLF4$^{-/-}$ mice can be correlated with the *in vitro* experiments where KC was not detectable in blood neutrophils (of KLF4$^{-/-}$ mice) on exposure to *S. pneumoniae* (see Fig. 13). However Clausen et al. have also suggested that the LyzMcre is able to cause conditional gene knockout in CD11c^{+} splenic DCs[170]. It has previously been reported that human CD11c^{+} DCs shows increased cytokine levels during infection with pneumococci such as TNF-α, IL-1β, IL-10, IL-8 (murine homolog: KC)[216]. Previous reports have also suggested that the LyzMcre was effective in knockdown of RelA protein from alveolar macrophages[217]. It has also been known that alveolar macrophages plays an anti-inflammatory role in the innate immune responses during pneumococcal pneumonia[218]. Whether KLF4 has any impact on the cytokine production of CD11C^{+} DCs and the alveolar macrophages during pneumococcal pneumonia needs to be addressed in further experiments.

With the production of less cytokine, it was expected that there would have been less neutrophil recruitment in the BALF and in the plasma[91,166]. However, in the present study, a less neutrophil recruitment was seen only in the blood but not in the BALF as given in Fig. 17. So what could be the reason that there were no differences in cell recruitment in the BALF? Mouse albumin ELISA was done to assess the vascular permeability within the lung. KLF4$^{-/-}$ mice showed an increased vascular permeability, which could be the reason that there are no changes in cell recruitment in the BALF, 24h after infection. It has earlier been reported that *S. pneumoniae* is responsible for increasing the vascular permeability within the lung[176] and that this increased permeability is associated with intra-alveolar edema[180]. The findings from histopathology analysis revealed that KLF4$^{-/-}$ mice, 24h after infection showed significantly higher levels of perivascular edema and pleuritis. Experiments with human lung tissue indeed revealed that *S. pneumoniae* induces oxidative stress and extensive tissue damage[219]. Thus, the higher bacterial load may be responsible for disrupting the lung architecture (as seen in the enhanced vascular permeability) and this leads to a higher perivascular edema and pleuritis[180,220]. All this taken together can suggest that why there no differences in cell recruitment in the BALF between the KLF4$^{-/-}$ and KLF4$^{+/+}$ mice. KLF4$^{-/-}$ mice also showed higher tendency of necrosis and more affected lung area in comparison to KLF4$^{+/+}$ mice. It is noteworthy to mention that it has already been reported that *S. pneumoniae* is one of the causes of necrosis in the human lung[221].

Sepsis is a condition in which infection in the blood can be documented[222]. From the blood CFU experiments, it was seen the *S. pneumoniae* could be detected in KLF4$^{+/+}$ mice and to a significantly higher extent in the KLF4$^{-/-}$ mice. Therefore, the 24h time point could be considered as a stage where the mice just started entering the state of sepsis. Certain groups have already pointed out that *S. pneumoniae* affects the myocardium of the heart and is also associated with acute kidney injury (AKI)[223,224]. Additional reports have also suggested that *S. pneumoniae* is also responsible for meningitis and sometimes gastrointestinal infections[49,50,225]. The hypothesis was that the spread out bacteria from the lungs in the blood could affect other organs too. Various organs such brain, thymus, heart, liver, spleen, small intestine, large intestine and the kidneys were isolated from the mice and analyzed for histopathology. However, no such impact could be found on these above-mentioned organs. The differences is *S. pneumoniae* serotype strain could be the reason that the results could not be validated with reported literature. Previous report have suggested that of the 90 different serotypes of *S. pneumoniae*, some strains are more prone to nasopharyngeal colonization while other strains are responsible for invasive disease and spread into the blood stream. In this context, serotype 4 have been reported to have high level of invasiveness and can be detected to a large extent in the blood[226,227,228]. In the previous study, where *S. pneumoniae* could induce cardiac micro lesion, the study was done using TIGR4 (serotype 4 strain) while the present study is done using serotype 3 strain.

When the progression of pneumonia was monitored over the period of 10 days, it was found that KLF4$^{-/-}$ mice had to be euthanized earlier in comparison to KLF4$^{+/+}$ mice. There was a significant difference between the survival of KLF4$^{-/-}$ and KLF4$^{+/+}$ mice. Various clinical symptoms associated with more sickness of KLF4$^{-/-}$ mice included raised fur, accelerated breathing rate and lower responses to external stimuli. As given in Fig. 20, it was seen that KLF4$^{-/-}$ mice had lower body temperature than KLF4$^{+/+}$ mice as early as day 2. There was however no significant differences between the body weight of KLF4$^{-/-}$ and KLF4$^{+/+}$ mice. The higher mortality of KLF4$^{-/-}$ mice in comparison to KLF4$^{+/+}$ mice during *S. pneumoniae* induced pneumonia are in line with the findings from Shen et al.: they had reported earlier that KLF4 KO mice have lower survivability with respect to WT mice after infection with *E. coli*[166]. Previous reports have described that in order to understand the progression of pneumococcal pneumonia in a murine model, the mice have to be monitored 10 days after infection[177,229]. It has already been reported that various clinical symptoms associated with infection in mice include fall in body temperature, less responsiveness, dyspnea[230]. Taken

together, the loss of myeloid KLF4 reduce the chances of survivability during pneumococcal pneumonia.

7.3 Concluding remarks

From the *in vitro* experiments, it was revealed that KLF4 is not expressed in basal conditions but there was an induction of KLF4 in neutrophils during pneumococcal infection. The expression of KLF4 was much higher with the unencapsulated pneumococci than the encapsulated, suggesting that the component for KLF4 induction may be partly hidden by the capsule. However, this expression of KLF4 is independent of TLR2 and TLR4 so it may not be induced by lipoteichoic acid, lipoprotein or Ply. Additionally, this expression of KLF4 was reduced in pneumococci strain deficient in autolysin and not induced by bacterial DNA alone. However, the combination of bacterial DNA and autolysin-deficient *S. pneumoniae* strain could partly induce the expression of KLF4, suggesting the expression of KLF4 in neutrophils depends on autolysis and at least partly on the sequential release of bacterial DNA. The bacterial DNA is probably recognized through an unknown DNA receptor independent of or additionally to TLR9. Although KLF4 has no role in the phagocytosis by neutrophils but it can upregulate pro-inflammatory cytokines such as TNF-α and KC which might be through the regulation of NF-κB pathway as have been reported earlier [136,167].

From the *in vivo* experiments it was found that $KLF4^{-/-}$ in myeloid cells led to higher bacterial load in the lung, blood, spleen of $KLF4^{-/-}$ mice with respect to $KLF4^{+/+}$ 24h post infection. The higher bacterial load was due to less cytokine production such as TNF-α, IL-1β, KC in the BALF and in the plasma. Though there was less cell recruitment in the blood of $KLF4^{-/-}$ mice, yet there were no differences in the cell recruitment in the BALF between $KLF4^{-/-}$ and $KLF4^{+/+}$ mice. The higher bacterial load in the $KLF4^{-/-}$ mice increased vascular permeability associated with perivascular edema, pleuritis and necrosis, which disrupted the lung architecture. The disrupted lung architecture allows the entry of non-functional myeloid cells, which cannot produce any cytokine (as seen in the *in vitro* data) for orchestrating the bacterial clearance. All this taken together could lead to an earlier onset of sepsis and an earlier death (euthanized) of $KLF4^{-/-}$ mice as seen from the survival data and clinical symptoms. The summary is shown in Fig. 22.

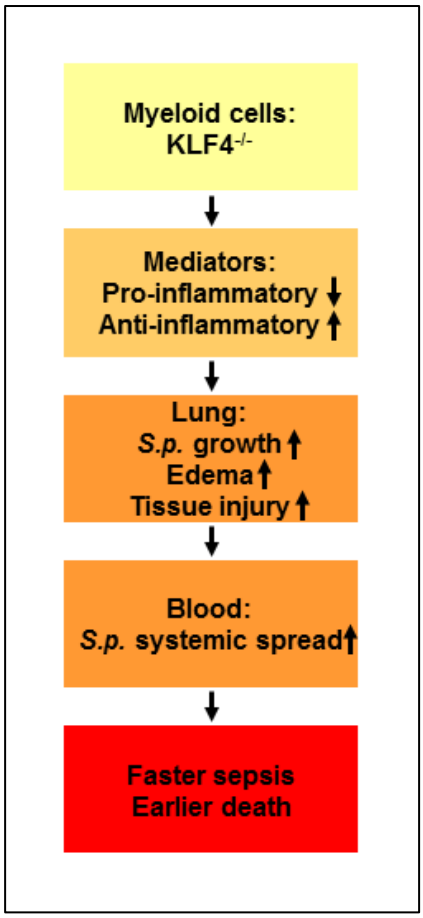


Fig. 22 Summary

7.4 Outlook

This study together with previous reports indicated that the transcription factor KLF4 must be considered as a major regulator of the (innate) immune response at least in pneumococcal pneumonia. As an outlook for the presented research project, in further studies, it should be investigated which pathways are responsible for the induction of KLF4 during bacterial pneumonia. The TLR data from this study should be verified by using e.g. neutrophils from TLR and other receptor (such as NLRs) KO mice. Deciphering the exact pathway through which KLF4 is expressed in neutrophils and how it can regulate the pro-inflammatory cytokine by acting directly on the NF-κB pathway or via other signaling pathways is also needed. This could be done for example by using chemical inhibitors of NF-κB-related pathways or knockdown experiments by siRNA, shRNA, CRISPR/Cas9 or other gene editing methods. Another important question is the impact of the different myeloid cells concerning the phenotype in the murine mouse model. To study this a transfer of isolated KLF4 KO cells (for example macrophages, neutrophils and DCs) in irradiated WT mice lacking immune cells could be done in future experiments.

This better understanding will help us develop novel therapies by targeting KLF4 as a central modulator of the inflammatory responses of myeloid cells during pneumococcal pneumonia. However, it should be noted that KLF4 is an important cell cycle regulator and also has been reported for its role in acute myeloid leukemia (AML) and therefore manipulation of myeloid KLF4 by e.g. adenoviral gene manipulation could bear the risk of the development of blood cancer[231].

Given that the research project is based on a murine model, the present study should be further investigated using pneumonia patient samples. There have been reports of differences in hematological parameters between human and mice[232]. Additionally, differences also exist between human and mice with respect to the innate and adaptive immune system[74]. As a result, neutrophils isolated from pneumococcal pneumonia patients should be checked for KLF4 expression in comparison to normal healthy subjects. The KLF4 expression in the neutrophils can be further correlated with chances of survival of the pneumonia patients. Given the poor outcome from extrapolation of rodent studies to humans[233], various reports also suggest the use of human lung tissue as model for studying the pathophysiology of lung diseases[234,235]. Taken together, the human *ex vivo* lung model should be used along with the patient samples to validate the functional role of KLF4 during pneumococcal pneumonia in human subjects.

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9 APPENDIX

9.1 List of figures

9.2 List of tables